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(71) Applicant *(for all designated States except US)*: EPIGENESIS PHARMACEUTICALS, INC. [US/US]; 2005 East Park Boulevard, Cranbury, NJ 08512 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: NYCE, Jonathan, W. [US/US]; 59 Sayre Drive, Princeton, NY 08540 (US).

(74) Agent: AMZEL, Viviana; Arter & Hadden LLP, Suite 3400, Citicorp Plaza, 725 S. Figueroa Street, Los Angeles, CA 90017–5434 (US).

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(54) Title: METHOD FOR VALIDATING/INVALIDATING TARGET(S) AND PATHWAYS

(57) Abstract

A method of determining the existence of a correlation between a function of a disease or condition and a gene or mRNA encoding a target polypeptide suspected of being associated with a disease or condition, comprises obtaining oligonucleotides (oligos) consisting of up to about 15 % adenosine (A), preferably having no adenosine content, and which is anti–sense to a target selected from the group consisting of target genes and their corresponding mRNAs, genomic and mRNA flanking regions selected from the group consisting of 3' and 5' intron–exon borders and the juxta–section between coding and non–coding regions, and all mRNA segments encoding polypeptides associated with a pre–selected disease or condition; selecting amongst the oligos one that significantly inhibits or ablates expression of the polypeptide encoded by the mRNA upon in vitro hybridization to the target mRNA; administering to a subject an amount of the selected oligo effective for in vivo hybridization to the target mRNA; and assessing a subject=s function that is associated with the disease or condition before and after administration of the oligo; wherein a change in the function=s value greater than about 70 % indicates a positive correlation, between about 40 and about 70 % a possible correlation, and below about 30 % a lack of correlation. The present method preferably administers the oligos in situ where the target is located, e.g. into the subject=s respiration when validating targets associated with malignant and other pulmonary and respiratory functions, so that the agent has direct access to the lungs. Alternatively, such desAdenosine oligos may be delivered directly to the CNS or other organs, tissues and organ systems, by means of known delivery formulations.

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METHOD FOR VALIDATING/INVALIDATING TARGET(S) AND PATHWAYS

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to a method for validating target genes, e. g. central nervous (CNS) genes which utilizes anti-sense oligonucleotides (oligos) of low adenosine (low A) or no adenosine (desA) content. This method aids in the screening of target genes and their functions by inhibiting the target gene's production of its product. This method is particularly suited for in vivo applications since many of a subject's functions are responsive to adenosine, and adenosine's effects may mask the specific target gene's detectable functions.

Description of the Background

The sequence of all human genes, approximately 100,000, is expected to become known within the next 1-3 years. This information will provide the opportunity to design new medicines for treatment of virtually all diseases which have in the past or presently afflict mankind. This massive accumulation of sequences, however, will not by itself be useful in the development of new medicines until the technology becomes available to discern the function of these genes, particularly with respect to the potential effects, i.e. therapeutic or toxicologic, of attenuating their function. For example, it is imperative that novel methods be designed for rapidly and accurately testing the function and, therefore, usefulness of newly discovered gene products as "targets" for drug discovery programs. In order to conserve drug discovery resources, such "targets" must be validated or invalidated as early as possible in the drug discovery process. Even so, the implementation of such methods will require the massive implementation of drug discovery programs to series of genes for which a sequence but not a function are known. One method of considerable potential to assess the value of newly discovered gene products as focal points for drug discovery programs is the use of anti-sense oligonucleotides to ablate the target gene function in vitro or in vivo. While this method has great theoretical importance, one problem is that anti-sense oligonucleotides have the potential to be degraded in vitro or in vivo, releasing their constitutive nucleotides. There is evidence that one of these products of oligonucleotide degradation, adenosine, is highly bioactive in certain tissues. Adenosine mediates pleiotropic effects including depression of neurotransmission, maintenance of thalamic spindle rhythms, sleep induction, antagonism of D1 and D2 dopamine receptors, anti-nociception, mediation of various effects of ethanol including motor incoordination, autonomic control of cardiac function, bronchoconstriction, negative chronotropy, inotropy and dromotropy, anti-β-adrenergic action, and renal sodium retention. Clearly, the liberation of even minute amounts of adenosine in certain tissues, e.g. CNS, the hyper-responsive asthmatic lung, heart, and kidney, among others, could locally activate adenosine receptors. This would make it impossible to interpret target validation data in a reliable, unambiguous fashion. Anti-sense oligonucleotides containing adenosine, thus, are not optimal to provide clear target validation data since their breakdown could cause pleiotropic adenosine-mediated effects.

Basic neuroscience research during the past few years has established a relationship between excitatory amino acid (EAA) central nervous system (CNS) transmitters, such as glutamic and aspartic acid, and various pathological states, e.g. stroke and CNS trauma. For example, a major mechanism of neural tissue degeneration following cerebral ischaemia, stroke or trauma, seems to involve overactivity of the EAA system in the brain, i.e. excessive release of glutamate and aspartate. This process is called delayed excitatory toxicity, and certain neural cell populations are selectively sensitive to excitatory toxicity.

Adenosine, among other activities, has been found to inhibit the release of EAA pre-synaptically and

thus attenuate this excitatory toxicity, and its release would greatly interfere with validation studies of this system. Because the effects of adenosine are generally mediated by extracellular receptors, the pharmacologically relevant pool of adenosine is, therefore, that which is outside the cell. Adenosine also has neuro-behavioral effects, and acts as a CNS depressant, i.e. inhibits neural activity. It is also a natural anti-convulsant and sedative. New studies are revealing that, under normal circumstances adenosine promotes sleep and, therefore, may interfere with pathways involved in sleep related responses, such as sleep apnea. Now increasing evidence is confirming that adenosine is an important "fatigue factor" and may also interfere with validation studies on the molecular underpinnings of the sleep cycle. For example in the brain, studies indicate that the key receptors reside on nerve cells in brain arousal networks. Some scientists believe that adenosine promotes sleepiness by targeting arousal networks in the brain such as the cholinergic system, such as the cholinergic basal forebrain and the mesopontine cholinergic nuclei which spur slumber. All these effects of adenosine interfere with any validation study of targets in the systems and pathways where it acts.

Accordingly, there is a definite need for a rapid and efficient method to screen large numbers of genes and their expression products to determine their functions and, thus, their usefulness in the design of therapeutic agents for treating diseases and conditions associated with the target genes and/or their expressed products. Moreover, there is a need for a method which is suitable for testing individual gene functions while avoiding triggering other gene functions which would obscure the interpretation of the results.

SUMMARY OF THE INVENTION

The present invention relates to a method of validating\invalidating or determining the existence of a correlation between a function of a disease or condition and, a gene or mRNA encoding a target polypeptide suspected of being associated with a disease or condition. The method generally comprises obtaining oligonucleotides (oligos) consisting of up to about 15% adenosine (A), and which is anti-sense to a target selected from the group consisting of target genes and their corresponding mRNAs, genomic and mRNA flanking regions selected from the group consisting of 3' and 5' intron-exon borders and the juxta-section between coding and non-coding regions, and all mRNA segments encoding polypeptides associated with a pre-selected disease or condition; selecting amongst the oligos one that significantly inhibits or ablates expression of the polypeptide encoded by the mRNA upon in vitro hybridization to the target mRNA; administering to a subject an amount of the selected oligo effective for in vivo hybridization to the target mRNA; and assessing a subject's function that is associated with the disease or condition before and after administration of the oligo; wherein a change in the function's value greater than about 70% indicates a positive correlation, between about 40 and about 70% a possible correlation, and below about 30% a lack of correlation. Suitable applications for the present method are in the elucidation of genes or networks of genes which may be associated with diseases or conditions afflicting the lung, brain, heart, kidney, tumor, blood, immune system, skin, eye, nasal passages, scalp, testes, cervix, oral cavity, pharynx, esophagus, intestine (small and large), synovir tissues, muscle, ovaries, and ear canal, among others, and in general any cells that contain, or originate from, the target sites.

The invention will now be described in reference to specific drawings. Other objects, advantages and features of the present invention will become apparent to those skilled in the art from the description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an experiment where saline (control), adenosine (o), and dAMP (P) were separately administered to rabbits. Saline had no effect, and both adenosine and dAMP affected a similar reduction compliance in a dose dependent manner. These results show that nucleosides such as dAMP either directly or following degradation and/or metabolism to adenosine, have the physiological effects of adenosine at

adenosine receptors.

Figure 2 accompanying this patent demonstrates that oligonucleotides (oligos) containing adenosine (A), but not those which do not have adenosine (desA) release bioactive adenosine. The released adenosine activates adenosine receptors and causes biological responses which may interfere with signals to be observed in validation studies. Here, two 21-mer randomer phosphorothioate anti-sense oligonucleotides (oligos), one containing adenosine (triangles) and one desA oligo (circles), were administered to asthmatic rabbits. The adenosine containing oligonucleotide caused a significant loss of airway compliance, reflecting A receptor activity while the desA randomer oligo did not.

Figures 3 and 4 demonstrate that anti-sense oligonucleotides may be utilized as effective agents in the validation of targets associated with pulmonary or airway diseases. Figure 3 illustrates the effects of oligonucleotides anti-sense to the adenosine A_1 receptor, and of mismatch control anti-sense oligonucleotides on the dynamic compliance of the bronchial airway in a rabbit model. Figure 3 illustrates the specificity of oligonucleotides anti-sense to the A_1 adenosine receptor as indicated by the A_1 and A_2 adenosine receptor number present in A_1 adenosine receptor anti-sense oligonucleotide-treated airway tissue.

The invention will be better understood in reference to the following description of the preferred embodiments.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention arose from a desire by the inventor to provide a novel technology for the rapid and efficient discovery of the function and, therefore, usefulness, of target genes and their products. The inventor surmised that he might successfully do this by applying his own prior discovery that low adenosine anti-sense oligonucleotides (oligos) may be administered in vivo to subjects without eliciting undesirable side effects mediated by adenosine receptors. The present method enables the assessment of the value of newly discovered genes and gene products as focal points for drug discovery programs with the aid of anti-sense oligonucleotides that ablate their function in vitro and/or in vivo. While the utilization of anti-sense oligonucleotides to ablate gene expression has had theoretical importance in the past, the inventor's finding that anti-sense oligonucleotides are degraded in vitro and in vivo, and release their constitutive nucleotides and nucleosides has hampered their successful application, explaining their ineffectiveness. The present inventor has evidence that one of the oligonucleoside degradation products, adenosine monophosphate is, as adenosine itself, highly bioactive in various tissues. Adenosine itself is known to mediate pleiotropic effects including depression of neurotransmission, sleep induction, antagonism of D1 and D2 dopamine receptors, anti-nociception, mediation of various effects of ethanol including motor incoordination, autonomic control of cardiac function, bronchoconstriction, negative chronotropy, inotrpy and dromotropy, anti-β-adrenergic action, and renal sodium retention, among others. Clearly, the liberation of even minute amounts of adenosine and as shown in the present examples adenosine nucleosides in certain tissues, e.g. CNS, lung, heart, and kidney, among others, could activate adenosine receptors in the local environment. This would make it impossible to interpret target validation data in a reliable, unambiguous fashion. Examples 30 and 31 and figures 1 and 2 accompanying this patent illustrate the break down of adenosine-containing oligonucleotides to release bioactive adenosine nucleosides. The figures show that oligonucleotides (oligos) containing adenosine (A), but not those without adenosine (desA), release bioactive adenosine. In the exemplary disclosure, two 21-mer randomer phosphorothioate anti-sense oligonucleotides (oligos), one containing adenosine (>) and one desA oligo (o), were administered to asthmatic rabbits. The results showed that the adenosine containing oligonucleotide caused a significant loss of airway compliance, reflecting adenosine receptor activity, while the desA randomer oligo did not. See, Example 31 below. In addition, an adenosine

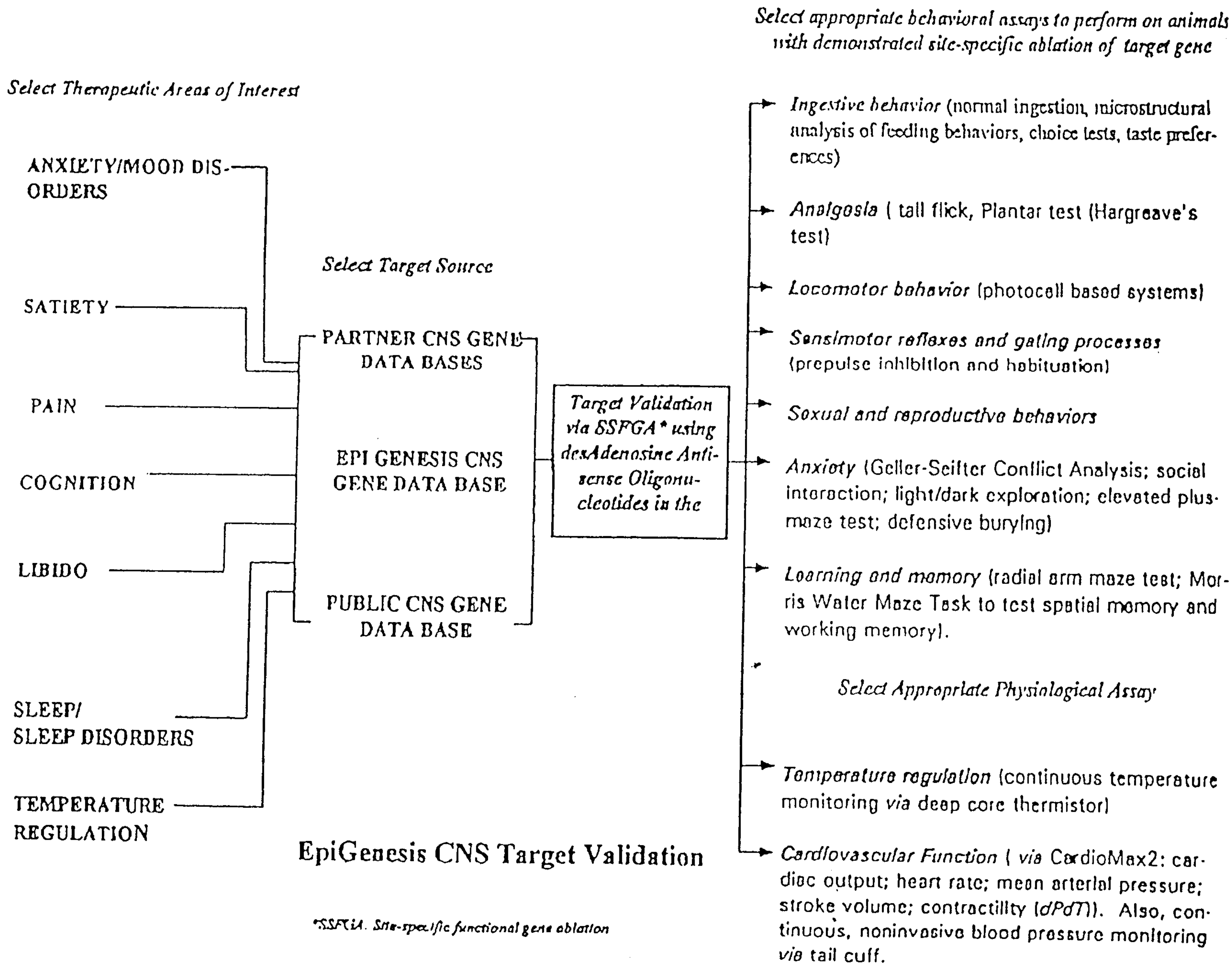
nucleoside (dAMP) was shown to have an effect similar to adenosine at an adenosine receptor. See, Example 30 below.

The work described here and results discussed in the examples accompanying this patent clearly shows that target validation is successfully attained by applying the method of the invention to new targets as they are discovered and/or to known targets as they become associated with their functions. The experimental work further indicates that the present method was found to be highly selective and effective at validating/invalidating targets when employing non-phosphodiester anti-sense oligonucleotides specifically targeted, by countering or reducing effects mediated by the target proteins, and the like. That was shown for all the anti-sense oligos targeting an adenosine A_1 receptor mRNA, the 1 anti-sense oligo targeting an adenosine A_{2b} receptor mRNA, the 2 anti-sense oligos targeting an A_3 receptor mRNA, and the 1 anti-sense oligo targeting a bradykinin receptor, were shown to counter effects mediated by the specific adenosine receptors elicited by exogenously administered adenosine. The method of this invention, moreover, is specific in validating/invalidating the specifically selected target, and fails to inhibit other targets, as shown with the anti-sense oligos targeted to the adenosine A_1 and bradykinin genes and mRNAs. In addition, the results show that the method of the invention employing low or no adenosine containing oligos results in extremely low or non-existent deleterious side effects or toxicity. This represents 100% success in providing a method that is highly effective and specific in the validation of targets, as shown here for the respiratory system. This invention is broadly applicable in the same manner to all gene(s) and corresponding mRNAs encoding proteins of the respiratory/pulmonary system, involved in or associated with airway diseases as well as targets of other systems associated with specific diseases or conditions which may be correlated with a specific function or end point, e.g. the CNS. A comparison was also made of the implementation of the method of the invention with the aid of a phosphodiester oligo, and a version of the same oligonucleotide wherein the phosphodiester bonds are substituted with phosphorothioate bonds. The results of the application of the method of the invention evidenced an unexpected superiority for the substituted over the phosphodiester oligonucleotides. Anti-sense oligonucleotides with a high content of adenosine (33%) thus are not suitable to attain clear target validation data since their breakdown could cause pleiotropic adenosine-mediated effects. The low adenosine oligomers utilized by the present method are clearly free of such side effects. This patent describes a method which may be utilized to perform unambiguous "target validation" that is enabling, for example, in the respiratory tract or pulmonary system, the CNS, and other organs or systems, where the liberation of adenosine may cause pleiotropic effects. The method involves the use of low A or desA anti-sense oligos, i.e. oligos that have a low A content or lack adenosine altogether, and which may not, therefore, liberate amounts of significant bioactive adenosine upon degradation. The present technology is enabled for practice with virtually any gene, but has particular applicability for the following gene subsets: G-protein Coupled Receptors, Neurohormone Receptors, Neuropeptide Receptors, Neurotransmitter Receptors, G Proteins, Calcium Channel Proteins, Sodium Channel Proteins, Potassium Channel Receptors, Chloride Channel Receptors, i.e. virtually any gene expressed in normal or diseased CNS, heart, lung, kidney, blood, immune system, and many more in addition to those listed below. Thus, the validation method described in this patent may also be applied not only to genes and systems mentioned here, but to other genuses and subgenuses of genes and gene networks.

Successful drug discovery programs depend on a fast, accurate assessment of the suitability of candidate gene products as drug design targets. Traditionally, this process has consumed an inordinate amount of time, personnel and financial resources. This invention provides a rapid, reliable method for target validation/invalidation in various biological systems that utilizes proprietary low or desAdenosine (desA) anti-sense oligonucleotides (collectively called here desA-ASONs). Using desA-ASONs, the present method may

validate/invalidate potential gene targets with a level of speed and accuracy that has heretofore been impossible using traditional technologies. DesA-ASONs provide greater levels of accuracy and speed vs. classical methods. DesA-ASONS cannot break down and release bioactive adenosine which would obfuscate target validation by causing adenosine-induced side effects. Among the ASONS there is a subgroup intended for respiratory administration herein referred to as RASONs. DesA-RASONs have been used by the inventor in practicing the method of this invention to validate adenosine A_1, A_2 and A_3 targets. The present technology is also applicable to CNS targets involving biochemical, behavioral, physiological assessments following site specific functional gene ablation in any region of the brain with desA-BASONs, brain anti-sense oligonucleotides (administered in situ in the brain) which cannot break down to release adenosine, a major modulator of brain physiology. In addition to the exemplary validation of pulmonary targets, the present method is illustrated below for CNS associated targets, and generally comprises performing the following several steps for target validation using desA anti-sense oligonucleotides.

The initial step requires the identification of a general system or area for the target validation, e.g. CNS, respiratory, renal, cardiac areas, etc. Then, the method turns to the aid of public libraries such as GenBank or other libraries in the public domain or private libraries such as those proprietary to specific companies. For example, a specific library such as one encompassing all G-protein coupled receptors (GPCRs) found in public and private data bases. Currently, there are about 250 GPCRs in existence. The present target validation method, thus, is suitable for testing what physiological, biophysical, biological, behavioral, etc., events occur when the selected group of targets, e.g. GPCRs, are individually attenuated using appropriate desA anti-sense oligos. For this, desA anti-sense oligos are designed as described below, and synthesized for pre-selected target genes, e.g. the GPCRs referred to above. The desA anti-sense oligos are then tested, first in vitro, for example by using an appropriate cell line, primary cell culture, or other cell tissues. Such in vitro testing may be applied to determine which of several desA anti-sense oligos designed against a specific target are "most active" as anti-sense agents. That is, which one best down regulates or ablates gene expression. This may be done by using a biophysical, biological, physiological, or other assay which may be correlated with a specific activity of the cell. In other cases, knocking out the target gene in the in vitro system may itself provide useful target validation information. The most active desA anti-sense oligos are then selected and applied in vivo, for example, by direct instillation into any brain region for CNS studies, by administration into the lung targets associated with the respiratory system, and the like. This may be done by methods known in the art, such as via stereotactic implantation of cannulae for brain targets, via inhalation for respiratory targets, systemically for blood targets, by in situ administration for organs and other localized systems and for both parenchymal cells and vascular heart cells, and systemically via inhalation or via direct instillation for renal targets. Behavioral, biophysical, physiological, biochemical, immunological, and other tests may be applied and data obtained on individual animals by administration of the appropriate anti-sense oligonucleotides to ablate one or more target genes. Behavioral functions or endpoints such as food ingestion, anxiety, libido, cognition, etc., or such physiological end points as temperature, electroencephalograms (ECG), electrocardiograms (EKG), glomerular filtration volume and content, ion retention, protein loss, etc., may be assessed by methods known in the art. These steps are illustrated for the CNS in the scheme shown below.



EpiGenesis CNS Target Validation

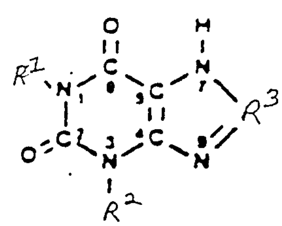
*SSFGA. Site-specific functional gene ablation

The present method relies on low A or desA-ASONs for application to respiratory (desA-RASONs), CNS (desA-BASONs), renal (desA-KASONs), cardiac (desA-CASONs), blood (desA-SASONs), immune system (desA-IASONs), malignant tissue (desA-MASONs), and other functions, without releasing significant bioactive amounts of adenosine. In this fashion, the present method prevents the undesirable activation of adenosine receptors in the lung, CNS, kidney, heart, blood, immune system, malignant cell aggregates, etc. Results obtained with presently available methods are less interpretable because standard anti-sense constructs contain normal (high) levels of adenosine about 25%, and activate adenosine receptors. This effect blurs the results and confuses the interpretation of functionality. In the lung, for example, oligo-released adenosine will cause changes in airway diameter (bronchoconstriction), inflammation, and secretion of surfactant, all effects that in the present case are unrelated to the validation of a different target. Such "side effects" make the data obtained with oligonucleotides or with ribozymes for that matter uninterpretable. Similarly when an A-containing anti-sense oligonucleotide is used in the brain, its effects obscure a plurality of functions which may be used as end points to validate targets. For example, adenosine causes depression of neurotransmission, sleep induction, anti-nociception, mediates various effects of ethanol including lack of motor coordination, autonomic control of cardiac function, antagonism of dopamine D1 and D2 receptors, alterations in CNS blood flow, and a host of other effects. The presence of significant amounts of adenosine clearly is contraindicated when anti-sense oligos are used in target validation studies in the hyper-responsive lung, CNS, and other systems which contain adenosine receptors or are otherwise responsive to adenosine. For these reasons, the present method provides a superior target validation tool. Particularly useful applications of the present method are to investigate therapeutic areas related to the respiratory tract, CNS, blood, malignant and uncontrollably growing cells, and many other systems, which may be separately targeted, and where one or more functions associated with the targets are separately measured.

As the present method has been invented at a singular time in the history of biological sciences, it will permit a faster and more efficient utilization of the information obtained from the sequencing of the human genome. Knowing the sequence of every gene in the human genome has been the Holy Grail of modern medicine. This accomplishment will enable the correlation of specific genes with their functions and thereafter the creation of entirely new types of medicines that strike closer to the heart of disease and lack the side effects of currently available drugs. The flood of sequences that is being banked currently provides important targets. The present method provides an invaluable tool to identify genes that are important in body function and, thus, in disease, and to determine whether or not the inhibition of their function is therapeutically useful. The process of determining if inhibiting the function of a gene is therapeutic is called here “Target Validation”. It is an important early step in the drug discovery process, and it helps determine whether or not critical resources are to be expended to develop new drugs for inhibiting the function of a target gene. In this context, it is just as important to validate a target as it is to invalidate it, by showing that inhibiting its function is without functional (therapeutic) effect. An early invalidation of a target in the drug discovery process prevents the waste of resources in unfruitful drug discovery campaigns. This, in turn, will permit a more rapid and focused investigation of more productive targets. The present rapid and accurate target validation method may make the difference between success and failure in the therapeutic application of large volumes of information, e.g. that was obtained from the human genome project. The in vivo testing of the anti-sense oligos in the method of the invention may be implemented, for example, in vitro and in animal models for important diseases, including models for respiratory diseases such as asthma, hormonal diseases, genetic diseases, obesity, and the like, including diseases of the CNS, renal, cardiac, blood diseases, etc. Assays known in the art may be utilized, such as whole body plethysmographic techniques in the conscious, unrestrained rodent, rabbit or primate, e.g. TruePrimateJ, or other species, applied to practice this

invention. The present method, thus, helps to determine the existence of a correlation between a function of a disease or condition and a gene or mRNA encoding a target polypeptide suspected of being associated with it. The method itself generally comprises obtaining oligonucleotides (oligos) consisting of up to about 15% adenosine (A), and which is anti-sense to a target selected from the group consisting of target genes and their corresponding mRNAs, genomic and mRNA flanking regions selected from the group consisting of 3' and 5' intron-exon borders and the juxta-section between coding and non-coding regions, and all mRNA segments encoding polypeptides associated with a pre-selected disease or condition; selecting amongst the oligos one that significantly inhibits or ablates expression of the polypeptide encoded by the mRNA upon in vitro hybridization to the target mRNA; administering to a subject an amount of the selected oligo effective for in vivo hybridization to the target mRNA; and assessing a subject's function that is associated with the disease or condition before and after administration of the oligo; wherein a change in the function's value greater than about 70% indicates a positive correlation, between about 40 and about 70% a possible correlation, and below about 30% a lack of correlation.

The anti-sense oligos may be constructed by selecting fragments of a target having at least 4 contiguous nucleic acids selected from the group consisting of G and C and obtaining a first oligonucleotide about 4, 6, 8, 10 to about 15, 25, 45, 60 nucleotides long which comprises the selected fragment and has a C and G content of about 0%, about 3%, about 5%, about 10%, about 12% up to about 15%. Alternatively, the target fragments may be selected by their type and/or extent of activity, which may vary for specific purposes. Any number of adenosines may be substituted, if present, from one to all, by a "universal" or alternative base such as heteroaromatic bases which bind to a thymidine base but have less than about 0.3 of the adenosine base agonist activity at the adenosine A_1, A_{2a}, A_{2b} and A_3 receptors, and heteroaromatic bases which have no activity at the adenosine A_{2a} receptor. The heteroaromatic bases may be pyrimidines and purines, which may be substituted, for example, by O, halo, NH_2, SH, SO, SO_2, SO_3, COOH and branched and fused primary and secondary amino, alkyl, alkenyl, alkynyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, alkoxy, alkenoxy, acyl, cycloacyl, arylacyl, alkynoxy, cycloalkoxy, aroyl, arylthio, arylsulfoxyl, halocycloalkyl, alkylcycloalkyl, alkenylcycloalkyl, alkynylcycloalkyl, haloaryl, alkylaryl, alkenylaryl, alkynylaryl, arylalkyl, arylalkenyl, arylalkynyl, arylcycloalkyl, which may be further substituted by O, halo, NH_2, primary, secondary and tertiary amine, SH, SO, SO_2, SO_3, cycloalkyl, heterocycloalkyl and heteroaryl. Other compounds and other substituents, however, are also suitable for use with the present method. Typically, the pyrimidines and purines are substituted at positions 1, 2, 3, 4, 7 and 8, although other substitutions are also encompassed. Examples of pyrimidines and purines are theophylline, caffeine, dyphylline, etophylline, acephylline piperazine, bamifylline, enprofylline and xanthine having the chemical formula



wherein R^1 and R^2 are independently H, alkyl, alkenyl or alkynyl and R^3 is H, aryl, dicycloalkyl, dicycloalkenyl, dicycloalkynyl, cycloalkyl, cycloalkenyl, cycloalkynyl, O-cycloalkyl, O-cycloalkenyl, O-cycloalkynyl, NH_2-alkylamino-ketoxyalkyloxy-aryl and mono and dialkylaminoalkyl-N-alkylamino-SO_2 aryl.

Specific examples of universal or alternative bases are 3-nitropyrrole-2'-deoxynucleoside, 5-nitro-indole, 2-deoxyribosyl-(5-nitroindole), 2-deoxyribofuranosyl-(5-nitroindole), 2'-deoxyinosine, 2'-deoxynebularine, 6H, 8H-3,4-dihydropyrimido [4,5-c] oxazine-7-one or 2-amino-6-methoxyaminopurine, although others are also suitable. Most preferred are adenosine analogs which have no activity at adenosine receptors, i.e., which have neither agonist nor antagonist properties at any adenosine receptor. The method may also utilize, in another preferred embodiment, a methylated cytocine (^{m}C) substituted in or for at least one unmethylated C in a CpG dinucleotide if present in the oligo(s), although many more or all may also be substituted. Other C-5 modifications at pyrimidines are also useful, e.g., C-5 propyne, among others. For practicing this method, one or more or all linking residues of the anti-sense oligonucleotides are preferably substituted or modified with a residue selected from the group consisting of methylphosphonate, phosphotriester, phosphorothioate, phosphorodithioate, boranophosphate, formacetal, thioformacetal, thioether, carbonate, carbamate, sulfate, sulfonate, sulfamate, sulfonamide, sulfone, sulfite, sulfoxide, sulfide, hydroxylamine, 2'-methylene(methyimino), (MMI), 2'-methoxymethyl (MOM), 2'-methoxyethyl (MOE), 2'-methyleneoxy (methylimino) (MOMA), 2'-methoxy methyl (MOM), 2'-O-methyl, phosphoramidate, and C-5 substitued (e. g. C-5 propyne) residues and combinations thereof. Anti-sense oligos suitable for practicing this method are about 7, about 9, about 11, about 13, about 15, about 18, about 21 to about 25, about 28, about 30, about 35, about 40, about 45, about 50, about 55, about 60 mononucleotides long, although other lengths are also suitable.

The method of the invention may also incorporate the use of an anti-sense oligo that is linked to an agent that is internalized or up-taken by cells as well as cell targeting agents, such as transferrin, asialoglycoprotein and streptavidin, among others known in the art. In one embodiment the oligo is linked to a vector, which may be prokaryotic or eukaryotic. Examples of vectors are known in the art and need not be described further in this patent. The amount of anti-sense oligo administered is generally one that is effective to reduce the production or availability, or to increase the degradation, of the mRNA, or to reduce the amount of the polypeptide present in situ. For example, when the gene to be validated is associated with a respiratory function, the anti-sense oligo may be administered directly to the lung (s). When the gene and the function are associated with another system, the nucleic acid is preferably administered in situ to the affected region, e. g. the brain, the heart, the kidney, the bladder, the gonads and the reproductive system in general, the respiratory and pulmonary systems, tumors in the case of cancer, the blood, the immune system, the lung, skin, eye, nasal passage, scalp, testes, cervix, oral cavity, larynx, esophagus, small and large intestine, synovial tissues, muscles, ovaries, ear canal, and many more, such as any cells that originate from a selected target site. In many cases, a disease or condition afflicts a certain system or area of a system, such as those described above. For example, a respiratory ailment may be associated with an increase in bronchoconstriction, inflammation, IgE-mediated allergies, surfactant production, and other symptoms such as in asthma, allergic rhinitis, COPD, lung tumors, ARDS, etc. Where a disease or condition is associated with an immunological dysfunction, the target may be selected amongst immunoglobulins and antibody receptors, cytokines and cytokine receptors, genes and other gene products, and corresponding mRNAs encoding them and other associated functions, the genes and mRNA flanking regions and intron and exon borders, among others. When the disease or condition is associated with a malignancy or cancer, the target may be selected from cancer related gene products, genes and mRNAs encoding them, genes and mRNAs associated with oncogenes, genomic and mRNA flanking regions and exon and intron borders, etc.

The anti-sense oligos for use with the present method may be produced by selection of a target from the group consisting of polypeptides associated with a disease(s) and/or condition(s) afflicting lung airways, genes and RNAs encoding them, the genomic and mRNA flanking regions and the gene(s) and mRNA(s)

intron and exon borders, then obtaining the sequence of a mRNA(s) selected from the group consisting of mRNAs corresponding to the target gene(s) and mRNAs encoding the target polypeptide(s), genomic and mRNA flanking regions and the genes and mRNAs intron and exon borders; selecting at least one segment of the mRNA(s), synthesizing one or more oligo anti-sense to the selected mRNA segment(s); and substituting, if necessary, a universal or alternative base(s) for one or more A(s) to reduce the content of A present in the oligo to up to about 10% of all nucleotides. Specific examples of the encoded polypeptides associated with the pulmonary system are NfκB Transcription Factor, Interleukin-8 Receptor (IL-8 R), Interleukin 5 Receptor (IL-5 R), Interleukin 4 Receptor (IL-4 R), Interleukin 3 Receptor (IL-3 R), Interleukin-1β (IL-1β), Interleukin 1β Receptor (IL- 1β R), Eotaxin, Tryptase, Major Basic Protein, β2-adrenergic Receptor Kinase, Endothelin Receptor A, Endothelin Receptor B, Preproendothelin, Bradykinin B2 Receptor, IgE High Affinity Receptor, Interleukin 1 (IL-1), Interleukin 1 Receptor (IL-1 R), Interleukin 9 (IL-9), Interleukin-9 Receptor (IL-9 R), Interleukin 11 (IL-11), Interleukin-11 Receptor (IL-11 R), Inducible Nitric Oxide Synthase, Cyclooxygenase (COX), Intracellular Adhesion Molecule 1 (ICAM-1) Vascular Cellular Adhesion Molecule (VCAM), Rantes, Endothelial Leukocyte Adhesion Molecule (ELAM-1), Monocyte Activating Factor, Neutrophil Chemotactic Factor, Neutrophil Elastase, Defensin 1, 2 and 3, Muscarinic Acetylcholine Receptors, Platelet Activating Factor, Tumor Necrosis Factor α, 5-lipoxygenase, Phosphodiesterase IV, Substance P, Substance P Receptor, Histamine Receptor, Chymase, CCR-1 CC Chemokine Receptor, CCR-2 CC Chemokine Receptor, CCR-3 CC Chemokine Receptor, CCR-4 CC Chemokine Receptor, CCR-5 CC Chemokine Receptor, Prostanoid Receptors, GATA-3 Transcription Factor, Neutrophil Adherence Receptor, MAP Kinase, Interleukin-9 (IL-9), NFAT Transcription Factors, STAT 4, MIP-1α, MCP-2, MCP-3, MCP-4, Cyclophillins, Phospholipase A2, Basic Fibroblast Growth Factor, Metalloproteinase, CSBP/p38 MAP Kinase, Tryptose Receptor, PDG2, Interleukin-3 (IL-3), Interleukin-1β (IL-1β), Cyclosporin A-Binding Protein, FK5-Binding Protein, α4β1 Selectin, Fibronectin, α4β7 Selectin, Mad CAM-1, LFA-1 (CD11a/CD18), PECAM-1, LFA-1 Selectin, C3bi, PSGL-1, E-Selectin, P-Selectin, CD-34, L-Selectin, p150,95, Mac-1 (CD11b/CD18), Fucosyl transferase, VLA-4, CD-18/CD11a, CD11b/CD18, ICAM2 and ICAM3, C5a, CCR3 (Eotaxin Receptor), CCR1, CCR2, CCR4, CCR5, LTB-4, AP-1 Transcription Factor, Protein kinase C, Cysteinyl Leukotriene Receptor, Tachychinnen Receptors (tach R), IκB Kinase 1 & 2, STAT 6, c-mas and NF-Interleukin-6 (NF-IL-6). Examples of polypeptides or genes associated with the CNS, ophthalmic, cardiovascular and cardiopulmonary systems are the family of G-protein coupled receptors (aproximately 250 known, and approximately 750-1,000 more postulated and yet to be sequenced), Neuropeptide genes, Neuropeptide receptor genes, Excitatory amino acid receptor genes, Chloride channel genes, Calcium channel genes, Purinergic receptor genes, Adrenergic receptor genes, Serotonin receptor genes, Serotonin transporter genes, Excitatory amino acid transporter genes, Potassium channel genes, Tyrosine kinases, Phosphorylases, Acetylcholine receptors, Cholecystokinin receptors, Nitric Oxide synthase, Dopamine receptors, Cholinergic receptors, Angiotensin, Angiotensin receptors, Ion Channels including Potassium Channels, Structural proteins including those related to myelination/demyelination and axonic and dendritic structures, Neurotransmitter release mediators and structures, Opthalmic disorders, especially of the retina and asociated structures, Calcitonin and its receptors, Calcineurin and its receptors, CGRP and its receptors, Atrial natriuretic peptide and its receptors, Brain natriuretic peptide and its receptors, Bradykinin and its receptors, Baroreceptors, GABA/GABA receptors, Benzodiazepine receptors, Cholinesterases, Cannabinoid receptors, Calmodulin and its receptors, Calcium/Sodim exchange pump

Carbonic anhydrase, Catecholamines and their receptors, Histamine receptors, Muscarinic receptors, Opioid receptors, Chemokines, Choline acetyltransferase, Cholecalciferol, Mediators of inflammation including

cytokines, interleukins, interferons and their receptors, enzymes of the lipoxygenase pathway, proteases, DP(PGD2) receptors, Inositol phosphate associated enzymes, Endothelins and their receptors, Enkephalinase, Enkephalins, Benzodiazepine receptors, GABA transaminase, Galanin and its receptors, Gastrin releasing factor, Growth factors, Growth factor inhibitors, Cyclins, Nucleoside kinases, Nucleotide kinases, Oncogenes' Receptors, 5-hydroxytryptamine (5HT) receptors, ADH, IGF, Insulin receptors, Lactamases, Kainate receptors, Kallikreins and their recepotrs, Leukotriene-associated enzymes, Lipocortins, L-NMMA and receptors, Melanocyte stimulating hormone, Steroid transporters and metabolism enzymes and synthases, NMDA and receptors, Morphine receptors, MPTP, Neurokinins and their receptors, Nicotinic receptors, Phospholipases, Platelet activating factors, Signal transduction proteins, PDGF, Dynorphins, Prostacyclins, Prolactin and its receptors, Prolactin release inhibiting factor, Prohormones, Prostanoids, Prostaglandins and their receptors, Thrombin, Prothrombin, Pteroylglutamic acid and its receptors, Lysergic acid receptors, Snake and scorpion venom recertors, Renin angiotensin system components, Reverse transcriptase, Second messengers and associated enzymes, Sodium channels, Somatostatin and its receptors, Somatotropin and its receptors, Substance P, Substance k, Synatpic transmitters, Tachikinins, Tetrodotoxin receptors, Thromboxanes and their receotrs, Thyroid hormones, Hormones, Thyrotropin and receptors, Protirelin, T4, T3 Topoisomerases, Tumor necrosis factors and their receptrors, TGFs and their receptors, Xanthine oxidase, Viral messenger RNAs, Bacterial mRNAs, Oxytocin and its receptors, Chlecystokinin and its receptors, Vasoactive intestinal peptide and its receptors, Monoamine oxidase, Tyrosine-kinase linked receptors, and many more. Other specific target genes are, for example, G-proteins and G-protein coupled receptors, calcium channel proteins and associated protein receptors, sodium channel proteins and associated protein receptors, potassium channel proteins and associated protein receptors, and chloride channel proteins and associated protein receptors, neurotransmitters and neurotransmitter receptors, neurohormones and neurohormone receptors, neuropeptides and neuropeptide receptors, and many others including the ones listed throughout this patent. Other target genes are, for example, G-proteins and G-protein coupled receptor, calcium channel proteins and associated protein receptors, sodium channel proteins and associated protein receptors, potassium channel proteins and associated protein receptors, and chloride channel proteins and associated protein receptors, neurotransmitters and neurotransmitter receptors, neurohormones and neurohormone receptors, neuropeptides and neuropeptide receptors, and many others.

In the present method, the composition may be administered in vitro, orally, intracavitarily, intranasally, intraanally, intravaginally, intrauterally, intracranially, pulmonarily, intrarenally, intranodularly, intraarticularly, intraotically, intralymphatically, transdermally, intrabucally, intravenously, subcutaneously, intramuscularly, intratumorously, intraglandularly, intraocularly, intracranial, into an organ, intravascularly, intrathecally, by implantation, by inhalation, intradermally, intrapulmonarily, into the ear, onto the skin or scalp or cervix (e.g. topically), into the heart, by slow release, by sustained release and by a pump, and the like. Examples of target genes and mRNAs associated with different systems and diseases are genes and mRNAs encoding polypeptides such as transcription factors, stimulating and activating factors, cytokines and their receptors, interleukins, interleukin receptors, chemokines, chemokine receptors, endogenously produced specific and non-specific enzymes, immunoglobulins, antibody receptors, central nervous system (CNS) and peripheral nervous and non-nervous system receptors, CNS and peripheral nervous and non-nervous system peptide transmitters, adhesion molecules, defensines, growth factors, vasoactive peptides, peptide receptors and binding protein and genes and mRNAs corresponding to oncogenes. The administration of the oligo may be conducted with an oral formulation having a liquid carrier such as solutions, suspensions, and oil-in-water and water-in-oil emulsions, and/or may be administered as a powder, dragees, tablets, capsules, sprays, aerosols, solutions, suspensions and emulsions. When administered as a topical formulation, the carrier may

be selected from creams, gels, ointments, sprays, aerosols, patches, solutions, suspensions and emulsions. When the formulation is injectable, the carrier may be selected from aqueous and alcoholic solutions and suspensions, oily solutions and suspensions and oil-in-water and water-in-oil emulsions, among others. When as a rectal formulation, it may be in the form of a suppository, when in the form of a transdermal formulation, the carrier is selected from aqueous and alcoholic solutions, oily solutions and suspensions and oil-in-water and water-in-oil emulsions, although others are also suitable. The transdermal formulation, may be an iontophoretic transdermal formulation, wherein the carrier is selected aqueous and alcoholic solutions, oily solutions and suspensions and oil-in-water and water-in-oil emulsions, and the formulation may also contain a transdermal transport promoting agent, of which many are known in the art. Also suitable for prolonged administration are implantable capsules or cartridges containing the formulation. In this case, the carrier may also be selected from aqueous and alcoholic solutions and suspensions, oily solutions and suspensions and oil-in-water and water-in-oil emulsions, be a hydrophobic carrier, such as lipid vesicles or particles, e. g. liposomes made of N-(1-[2, 3-dioleoxyloxi] propyl) -N,N,N- trimethyl- ammonium methylsulfate. and/or other lipids, and microcrystals. For pulmonary applications, the formulation is preferably a respirable or inhalable formulation, e. g. in the form of an aerosol. For prolonged exposure of a target area, the oligo may be delivered through a localized implant, suppository, sublingual formulation, and the like, all of which are known in the art.

A factor which proves this method superior to other technology is the ability to obtain data of greater reliability and accuracy. Anti-sense ribozyme technology is unstable in in vivo environments, and the presence of adenosine in ribozyme and other oligonucleotides prevents the attainment of reliable data, for instance in the hyperactive respiratory tract, and other systems having a substantial number of adenosine receptors. No other method has proven, up to the present time, capable of unambiguously attenuating targets in A-containing systems, e.g. the respiratory tract while providing reliable and accurate correlations. In addition, the present method may be utilized to the elucidation of gene networks, e.g. neuronal gene networks, a quantum leap above the identification of single genes in isolation of their broader context. This may be done by selecting more than one target linked in a metabolic pathway and testing them separately and in conjunction with the others, that is by administration of one anti-sense oligo at a time, then in twos, in threes, etc., and comparing the results to ascertain whether or not there is linkage, they work sequentially etc. The present method permits the creation of a gene network data base suitable to supplement a more elaborate pharmoincentive discovery process to meet critical medical needs in areas such as cognition, memory, pain, anxiety, behavioral disturbances, ingestive behavior, hunger and satiety, and neurological disease, among others relating to the CNS. The present technology encompasses four basic areas: functional genomics applied to discerning neuronal gene networks of relevance to new drug discovery, in situ hybridization to understand the distribution of these networks within the brain, proprietary Site Specific Functional Gene Ablation (SSFGA) to determine the function of individual genes within network, Multifactorial Behavioral Analysis for qualitative and quantitative analysis of the participation of genes and gene networks in various behaviors of medical relevance, and physiological, biophysical, biochemical, etc. analysis to discern the effects of genes and gene networks upon extrapyramidal systems such as the cardiovascular and pulmonary systems.

The application of this method to the CNS may encompass areas such as pain, satiety, anxiety/mood disorders, libido, cognition/cognitive disorders, and sleep/sleep disorders, among many others.The ability to discern functional significance of identified novel genes and gene networks relies on an assessment of their spatial representation. In situ hybridization, for example, may be is used to determine the precise three dimensional (3-D) location of selected genes and gene networks within the brain, and to enable the accurate

targeting of gene ablation studies for assessing their significance as candidates for drug discovery programs. Site-specific functional gene ablation (SSFGA) provides a means to selectively attenuate the expression of any target gene in any desired region of a system, e.g. the brain. SSFGA for CNS target validation may be performed with the aid of the low A or desA anti-sense oligonucleotide designed as described below. Other approaches utilizing anti-sense oligonucleotides provide ambiguous data because the oligonucleotides used breakdown and release adenosine, one of the most bioactive autocoids, e.g. in the CNS and in other target systems containing adenosine receptors. The release of adenosine upon break-down of oligonucleotides either depresses or facilitates neurotransmission depending upon the system and the specific area, e.g. the brain region, where it is released, induces sleep, affects nociception, alters thalamic spindle rhythms, mitigates or potentiates myriad drug effects, affects autonomic control of cardiovascular function and respiration, inhibits Ca+ currents and presynaptic function of GABA, depresses both spontaneous and evoked neuronal firing, inhibits the release of neurotransmitters, decreases postsynaptic excitability, inhibits long-term potentiation postulated to be an underlying event in learning and memory, and induces pleiotropic effects by causing cephalic bronchodilation. Clearly, the release of significant amounts of adenosine via break down of oligonucleotides in the brain, or elsewhere, of the experimental animal is contraindicated in target validation studies. Neurological and behavioral tests suitable for application as end points for CNS target validation are known in the art. For example, tests for memory, three dimensional or spatial ability, cognition, motor control, sensitivity and responsiveness to exogenous triggers, vision, eye coordination, skin sensory ability, gustation and olfactory recognition, and many more. The testing of physiological parameters is also known in the art and may rely on the measurement of electrical conductivity such as EKGs and EEGs, or other bodily functions such as heart rate and rhythmicity, water voiding, sleep patterns, etc. In many cases, side effects, particularly cardiopulmonary, renal, and other side effects, constitute a major reason for disqualifying potential drug discovery CNS targets. And vice versa, CNS side effects often disqualify otherwise suitable therapeutic cardiopulmonary, renal, and other agents. It thus would be advantageous to obtain evidence of cardiopulmonary effects induced by attenuation of CNS targets as early as possible in the development of a drug. When such effects are discovered late in a development program they cause the cancellation of a program at a much later stage. The present method utilizes state of the art analysis, significant surgical, electrophysiological, and other types of tests, to assess any detrimental effects, e.g. cardiovascular effects of attenuating respiratory targets, and vice versa any detrimental side effects of attenuating respiratory targets on CNS or the cardiovascular system, among others. For example, lung function studies performed in conscious, unrestrained animals, and cardiac function studies may provide further insight on potential effects of attenuating candidate CNS targets.

Target validation may proceed via SSFGA as described above using low A or desA anti-sense oligonucleotides targeting a variety of known receptors with suspected function in human pathology, e.g. neuropeptide Y (NPY)/leptin receptors and ingestive behavior, as well as novel targets found from CNS gene libraries. Animal models may be subjected to SSFGA targeting of a specific receptor and then assessed for changes in various behaviors, such as in ingestive behavior, analgesia, locomotor behavior, sensimotor reflexes, gating processes, sexual and reproductive behaviors, anxiety, learning and memory, among many others. This broad-based behavioral battery of tests provides a sensitive measure of the effect of attenuating specific CNS targets, and a critical knowledge in the initial stages of the drug development decision process. Combined with a physiological/biophysical/biochemical analysis, this method provides for a rapid, intensive determination of the potential of a candidate target as a worthwhile focus for further drug discovery efforts. The present method improves on prior methods for validation of gene and protein targets in that desadenosine anti-sense oligos targeted to genes associated with different systems are used to inhibit the expression of a

gene product and to test the effects and symptomatology and changes these procedures. The present invention is premised on the recent discovery by the inventor that, when oligonucleotides are metabolized in vivo to their mononucleotides, bioactive adenosine metabolites are released. Adenosine (A)-containing oligonucleotides break down and release adenosine metabolites which, in turn, activate adenosine receptors which, for example, in the lungs cause bronchoconstriction, inflammation, and the like. The present technology relies on the design of anti-sense oligos targeted to genes and mRNAs associated with systems involved in different functions, ailments, and pathology(ies). The oligos are modified to reduce their adenosine content to minimize the occurrence of undesirable side effects caused by its release upon breakdown. Doing so improves the statistical significance of the results observed, particularly where adenosine receptors may be involved in effects similar, or opposite to those being observed, or indirectly by producing changes in the experimental model's homeostasis of the system being observed. In this manner, the inventor targets a specific gene to design one or more anti-sense oligonucleotide(s) (oligos) that selectively bind(s) to the corresponding mRNA and, if necessary, reduces their content of adenosine via substitution with universal or alternative base or an adenosine analog incapable of activating adenosine A_1, A_{2a}, A_{2b} or A_3 receptors. Based on his prior experience in the field, the inventor reasoned that in addition to "downregulating" or ablating specific genes, he could increase the accuracy of the results by either selecting segments of RNA that are devoid, or have a low content, of thymidine (T) or, alternatively, substitute one or more adenosine(s) present in the designed oligonucleotide(s) with other nucleotide bases, so called universal or alternative bases, which bind to thymidine but lack the ability to activate adenosine receptors and otherwise exercise the effect of adenosine in the lungs, etc. Given that adenosine (A) is a nucleotide base complementary to thymidine (T), when a T appears in the RNA, the anti-sense oligo will have an A at the same position. For consistency's sake, all RNAs and oligonucleotides are represented in this patent by a single strand in the 5' to 3' direction, when read from left to right, although their complementary sequence(s) is (are) also encompassed within the four corners of the invention. In addition, all nucleotide bases and amino acids are represented utilizing the recommendations of the IUPAC-IUB Biochemical Nomenclature Commission, or by the known 3-letter code (for amino acids).

The method of the present invention may be used to validate or invalidate any number of target genes, from single genes associated with one function in a subject, to multiple single genes associated with a network or pathway within a system. By validation/invalidation it is meant a demonstration through experimental evidence whether a specific gene is involved in any one of a number of biophysical, biochemical, physiological, behavioral, etc., functions. Even if a gene appears initially not to have an effect, it may be tested in conjunction with another gene target, as there may be a requirement for the simultaneous obliteration of their expression to observe an effect. The adenosine content of the anti-sense agent(s) of the invention have a reduced A content to prevent its liberation upon in vivo degradation of the agent(s). For example, if the system is the pulmonary or respiratory system, a large number of genes is involved in different functions, including those listed in Table 1 below.

Table 1: Pulmonary Disease or Condition Pulmonary and Inflammation Targets

NfκB Transcription Factor	Interleukin-8 Receptor (IL-8 R)
Interleukin-5 Receptor (IL-5R)	Interleukin-4 Receptor (IL-4R)
Interleukin-3 Receptor (IL-3R)	Interleukin-1β (IL-1β)
Interleukin-1β Receptor (IL-1βR)	Eotaxin
Tryptase	Major Basic Protein
β2-adrenergic Receptor Kinase	Endothelin Receptor A

Endothelin Receptor B	Preproendothelin
Bradykinin B2 Receptor (B2BR)	IgE (High Affinity Receptor)
Interleukin-1 (IL-1)	Interleukin 1 Receptor (IL-1 R)
Interleukin-9 (IL-9)	Interleukin-9 Receptor (IL-9 R)
Interleukin-11 (IL-11)	Interleukin-11 Receptor (IL-11 R)
Inducible Nitric Oxide Synthase	Cyclooxygenase (COX)
Intracellular Adhesion Molecule 1 (ICAM-1)	Vascular Cellular Adhesion Molec.Subst.P (VCAM)
Rantes	Endothelial Leukocyte Adhesion Molecule
Endothelin ETA Receptor	(ELAM-1)
Cyclooxygenase-2 (COX-2)	GM-CSF, Endothelin-1
Monocyte Activating Factor	Neutrophil Chemotactic Factor
Neutrophil Elastase	Defensin 1,2,3
Muscarinic Acetylcholine Receptors	Platelet Activating Factor
Tumor Necrosis Factor α	5-lipoxygenase
Phosphodiesterase IV	Substance P
Substance P Receptor	Histamine Receptor
Chymase	CCR-1 CC Chemokine Receptor
Interleukin-2 (IL-2)	Interleukin-4 (IL-4)
Interleukin-12 (IL-12)	Interleukin-5 (IL-5)
Interleukin-6 (IL-6)	Interleukin-7 (IL-7)
Interleukin-8 (IL-8)	Interleukin-12 Receptor (IL-12R)
Interleukin-7 Receptor (IL-7R)	Interleukin-1 (IL-1)
Interleukin-14 Receptor (IL-14R)	Interleukin-14
CCR-2 CC Chemokine Receptor	CCR-3 CC Chemokine Receptor
CCR-4 CC Chemokine Receptor	CCR-5 CC Chemokine Receptor
Prostanoid Receptors	GATA-3 Transcription Factor
Neutrophil Adherence Receptor	MAP Kinase
Interleukin-15 (IL-15)	Interleukin-15 Receptor (IL-15R)
Interleukin-11 (IL-11)	Interleukin-11 Receptor (IL-11R)
NFAT Transcription Factors	STAT 4
MIP-1α	MCP-2
MCP-3	MCP-4
Cyclophillin (A, B, etc.)	Phospholipase A2
Basic Fibroblast Growth Factor	Metalloproteinase
CSBP/p38 MAP Kinase	Tryptase Receptor
PDG2	Interleukin-3 (IL-3)
Interleukin-10 (IL-10)	Cyclosporin A - Binding Protein
FK506-Binding Protein	α4β1 Selectin
Fibronectin	α4β7 Selectin
cMad CAM-1	LFA-1 (CD11a/CD18)
PECAM-1	LFA-1 Selectin
C3bi	PSGL-1
E-Selectin	P-Selectin
CD-34	L-Selectin
p150,95	Mac-1 (CD11b/CD18)
Fucosyl transferase	VLA-4
STAT-1	STAT-2
CD-18/CD11a	CD11b/CD18
ICAM2 and ICAM3	C5a
CCR3 (Eotaxin Receptor)	CCR1, CCR2, CCR4, CCR5
LTB-4	AP-1 Transcription Factor
Protein kinase C	Cysteinyl Leukotriene Receptor
Tachykinnen Receptors (tach R)	IκB Kinase 1 & 2
Interleukin-2 Receptor (IL-2R)	(e.g., Substance P, NK-1 & NK-3 Receptors)

STAT 6	c-mas
NF-Interleukin-6 (NF-IL-6)	Interleukin-10 Receptor (IL-10R)
Interleukin-3 (IL-3)	Interleukin-2 Receptor (IL-2R)
Interleukin-13 (IL-13)	Interleukin-12 Receptor (IL-12R)
Interleukin-14 (IL-14)	Interleukin-6 Receptor (IL-6R)
Interleukin-16 (IL-16)	Interleukin-13 Receptor (IL-13R)
Medullasin	Interleukin-16 Receptor (IL-16R)
Adenosine A_1 Receptor (A_1 R)	Tryptase-I
Adenosine A_{2b} Receptor (A_{2b} R)	Adenosine A_3 Receptor (A_3 R)
β Tryptase	STAT-3
Adenosine A_{2a} Receptor (A_{2a} R)	IgE Receptor β Subunit (IgE R β)
Fc-epsilon receptor CD23 antigen	IgE Receptor α Subunit (IgE R α)
IgE Receptor Fc Epsilon Receptor (IgERFcϵR)	Substance P Receptor
Histidine decarboxylase	Tryptase-1
Prostaglandin D Synthase	Eosinophil Cationic Protein
Eosinophil Derived Neurotoxin	Eosinophil Peroxidase
Endothelial Nitric Oxide Synthase	Endothelial Monocyte Activating Factor
Neutrophil Oxidase Factor	Cathepsin G
Macrophage Inflammatory Protein-1-Alpha/Rantes Receptor	Interleukin-8 Receptor α Subunit (IL-8 Rα)
	Endothelin Receptor ET-B

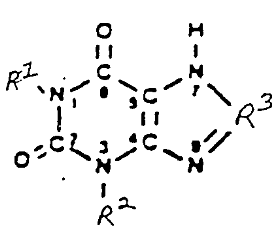
These genes, and others, are involved in the normal functioning of respiration as well as in diseases associated with respiratory pathologies, including cystic fibrosis, asthma, pulmonary hypertension and vasoconstriction, chronic obstructive pulmonary disease (COPD), chronic bronchitis, respiratory distress syndrome (ARDS), allergic rhinitis, lung cancer and lung metastatic cancers and other airway diseases, including those with inflammatory response. Anti-sense oligos to the adenosine A_1, A_{2a}, A_{2b}, and A_3 receptors, CCR3 (chemokine receptors), bradykinin 2B, CAM (vascular cell adhesion molecule), and eosinophil receptors, among others, have been shown to be effective in down-regulating the expression of their genes. Some of these act to alleviate the symptoms or reduce respiratory ailments and/or inflammation, for example, by "down regulation" of the adenosine A_1, A_{2a}, A_{2b}, and/or A_3 receptors and CCR3, bradykinin 2B, VCAM (vascular cell adhesion molecule) and eosinophil receptors. These agents may be utilized by the present method alone or in conjunction with anti-sense oligos targeted to other genes to validate pathway and/or networks in which they are involved. For better results, the oligos are preferably administered directly into the respiratory system, e.g., by inhalation or other means, of the experimental animal, so that they may reach the lungs without widespread systemic dissemination. This permits the use of low agent doses as compared with those administered systemically or by other generalized routes and, consequently, reduces the number and degree of undesirable side effects resulting from the agent's widespread distribution in the body. The agent(s) of this invention has (have) been shown to reduce the amount of receptor protein expressed by the tissue. These agents, thus, rather than merely interacting with their targets, e.g. a receptor, lower the number of target proteins that other drugs may interact with. In this manner, the present agent(s) afford(s) extremely high efficacy with low toxicity.

The receptors discussed above are mere examples of the high power of the present technology. In fact, a large number of genes may be targeted in a similar manner by practicing the present methods, to significantly down-regulate or obliterate protein expression and observe any changes wrought to one or more functions within a system, e.g. the respiratory, CNS, cardiovascular, renal and other systems. By means of example, in the respiratory system, the functions tested may be ease of breathing, bronchoconstriction, inflammation, chronic bronchitis, surfactant production, and the like, and others associated with diseases and conditions such as chronic obstructive pulmonary disease (COPD), inhalation burns, Acute Respiratory

Distress Syndrome (ARDS), cystic fibrosis, pulmonary fibrosis, radiation pulmonitis, tonsilitis, emphysema, dental pain, oral inflammation, joint pain, esophagitis, lung cancer and esophageal cancer, among others. These functions are of great interest because of their association with respiratory dysfunction, as is the case in asthma, allergies, allergic rhinitis, pulmonary bronchoconstriction and hypertension, chronic obstructive pulmonary disease (COPD), allergy, asthma, cystic fibrosis, Acute Respiratory Distress Syndrome (ARDS), cancer, which either directly or by metastasis afflict the lung, the present method may be applied to a list of potential target mRNAs, which includes the targets listed in Table 1 above, among others. In the CNS system, functions that may be selected are food ingestion/satiety, mood variation, anxiety, libido/sexual dysfunction, cognition, sexual function/dysfunction, brain trauma, Alzheimer's mediators, aneurism, etc.

The oligos of this invention may be obtained by first selecting fragments of a target nucleic acid having at least 4 contiguous nucleic acids selected from the group consisting of G and C and/or having a specific type and/or extent of activity, and then obtaining a first oligonucleotide 4 to 60 nucleotides long which comprises the selected fragment and has a thymidine (T) nucleic acid content of up to and including about 15%, preferably, about 12%, about 10%, about 7%, about 5%, about 3%, about 1%, and more preferably no thymidine. The latter step may be conducted by obtaining a second oligonucleotide 4 to 60 nucleotides long comprising a sequence which is anti-sense to the selected fragment, the second oligonucleotide having an adenosine base content of up to and including about 15%, preferably about 12%, about 10%, about 7%, about 5%, about 3%, about 1%, and more preferably no adenosine. When the selected fragment comprises at least one thymidine base, an adenosine base may be substituted in the corresponding anti-sense nucleotide fragment with a universal or alternative base selected from the group consisting of heteroaromatic bases which bind to a thymidine base but have less than about bout 10%, preferably less than about 1%, and more preferably less than about 0.3% of the adenosine base agonist activity at the adenosine A_1, A_{2a}, A_{2b} and A_3 receptors, and heteroaromatic bases which have no activity at the adenosine A_{2a} receptor, when validating in the respiratory system. Other adenosine activities in other systems may be determined in other systems, as appropriate.

The analogue heteroaromatic bases may be selected from all pyrimidines and purines, which may be substituted by O, halo, NH_2, SH, SO, SO_2, SO_3, COOH and branched and fused primary and secondary amino, alkyl, alkenyl, alkynyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, alkoxy, alkenoxy, acyl, cycloacyl, arylacyl, alkynoxy, cycloalkoxy, aroyl, arylthio, arylsulfoxyl, halocycloalkyl, alkylcycloalkyl, alkenylcycloalkyl, alkynylcycloalkyl, haloaryl, alkylaryl, alkenylaryl, alkynylaryl, arylalkyl, arylalkenyl, arylalkynyl, arylcycloalkyl, which may be further substituted by O, halo, NH_2, primary, secondary and tertiary amine, SH, SO, SO_2, SO_3, cycloalkyl, heterocycloalkyl and heteroaryl. The pyrimidines and purines may be substituted at all positions as is known in the art, but preferred are those which are substituted at positions 1, 2, 3, 4, 7 and/or 8. More preferred are pyrimidines and purines such as theophylline, caffeine, dyphylline, etophylline, acephylline piperazine, bamifylline, enprofylline and xantine having the chemical formula



wherein R^1 and R^2 are independently H, alkyl, alkenyl or alkynyl and R^3 is H, aryl, dicycloalkyl,

dicycloalkenyl, dicycloalkynyl, cycloalkyl, cycloalkenyl, cycloalkynyl, O-cycloalkyl, O-cycloalkenyl, O-cycloalkynyl, NH_2-alkylamino-ketoxyalkyloxy-aryl, mono and dialkylaminoalkyl-N-alkylamino-SO_2aryl, among others. Similar modifications in the sugar are also embodiments of this invention. Reduced adenosine content of the anti-sense oligos corresponding to the thymidines (T) present in the target RNA serves to prevent the breakdown of the oligos into products that free adenosine into the system, e.g. the lung, brain, heart, kidney, etc., tissue environment and, thereby, to prevent any unwanted effects due to it.

By means of example, the NfκB transcription factor may be selected as a target, and its mRNA or DNA searched for low thymidine (T) or desthymidine (desT) fragments. Only desT segments of the mRNA or DNA are selected which, in turn, will produce desA anti-sense as their complementary strand. When a number of RNA desT segments are found, the sequence of the anti-sense segments may be deduced. Typically, about 10 to 30 and even larger numbers of desA anti-sense sequences may be obtained. These anti-sense sequences may include some or all desA anti-sense oligonucleotide sequences corresponding to desT segments of the mRNA of the target, such as anyone of those shown in Table 1 above, in Table 2 below, and others associated with functions of the brain, cardiovascular and renal systems, and many others. When this occurs, the anti-sense oligonucleotides found are said to be 100% A-free. For each of the original desA anti-sense oligonucleotide sequences corresponding to the target gene, e.g. the NFκB transcription factor, typically about 10 to 30 sequences may be found within the target gene or RNA which have a low content of thymidine (RNA). In accordance with this invention, the selected fragment sequences may also contain a small number of thymidine (RNA) nucleotides within the secondary or tertiary or quaternary sequences. In some cases, a large adenosine content may suffice to render the anti-sense oligonucleotide less active or even inactive against the target. In accordance with this invention, these so called "non-fully desA" sequences may preferably have a content of adenosine of less than about 15%, about 12%, about 10%, about 7%, about 5%, and about 2% adenosine. Most preferred is no adenosine content (0%). In some instances, however, a higher content of adenosine is acceptable and the oligonucleotides still fail to show detrimental "adenosine activity". A particular important embodiment is that where the adenosine nucleotide is "fixed" or replaced by a "Universal or alternative" base that may base-pair with similar or equal affinity to two or more of the four nucleotide present in natural DNA: A, G, C, and T.

A universal or alternative base is defined in this patent as any compound, more commonly an adenosine analogue, which has substantial capacity to hybridize to thymidine, reduced, or substantially lacks ability to bind adenosine receptors or other molecules through which adenosine may exert an undesirable side effect in the experimental animal or in a cell system. Alternatively, adenosine analogs which completely fail to activate adenosine receptors, such as the adenosine A_1, A_{2a}, A_{2b} and/or A_3 receptors, most preferably A_1 receptors, may be used. One example of a universal or alternative base is α-deoxyribofuranosol-(5-nitroindole), and an artisan will know how to select others. This "fixing" step generates further novel sequences, different from those anti-sense to the ones found in nature, that permits the anti-sense oligonucleotide to bind, preferably equally well, with the target RNA. Other examples of universal or alternative bases are 2-deoxyribosyl-(5-nitroindole). Other examples of universal or alternative bases are 3-nitropyrrole-2'-deoxynucleoside, 5-nitro-indole, 2-deoxyribosyl-(5-nitroindole), 2-deoxyribofuranosyl-(5-nitroindole), 2'-deoxyinosine, 2'-deoxynebularine, 6H, 8H-3,4-dihydropyrimido [4,5-c] oxazine-7-one and 2-amino-6-methoxyaminopurine. In addition to the above, Universal or alternative bases which may be substituted for any other base although with somewhat reduced hybridization potential, include 3-nitropyrrole 2'-deoxynucleoside 2-deoxyribofuranosyl-(5-nitroindole), 2'-deoxyinosine and 2'-deoxynebularine (Glen Research, Sterling, VA). More specific mismatch repairs may be made using "P" nucleotide, 6H, 8H-3,

4-dihydropyrimido[4,5-c] [1,2] oxazin-7-one, which base pairs with either guanine (G) or adenine (A) and "K" nucleotide, 2-amino-6-methoxyaminopurine, which base pairs with either cytidine (C) or thymidine (T), among others. Others which are known in the art or will become available are also suitable. See, for example, Loakes, D. and Brown, D. M., Nucl .Acids Res. 22:4039-4043 (1994); Ohtsuka, E. et al., J. Biol. Chem.260(5):2605-2608 (1985); Lin, P.K.T. and Brown, D. M., Nucleic Acids Res. 20(19):5149-5152 (1992; Nichols, R. et al., Nature 369(6480): 492-493 (1994); Rahmon , M. S. and Humayun, N. Z., Mutation Research 377 (2): 263-8 (1997); Amosova, O., et al., Nucleic Acids Res. 25 (!0): 1930-1934 (1997); Loakes D. & Brown, D. M., Nucleic Acids Res. 22 (20): 4039-4043 (1994), the entire sections relating to universal or alternative bases and their preparation and use in nucleic acid binding being incorporated herein by reference.

When non-fully desT sequences are found in the naturally occurring target, they typically are selected so that about 1 to 3 universal or alternative base substitutions will suffice to obtain a 100% "desA" anti-sense oligonucleotide. Thus, the present method provides either anti-sense oligonucleotides to different targets which are low in, or devoid of, A content, as well as anti-sense oligonucleotides where one or more adenosine nucleotides, e. g. about 1 to 3, or more, may be "fixed" by replacement with a "Universal or alternative" base. Universal or alternative bases are known in the art and need not be listed herein. An artisan will know which bases may act as universal or alternative bases, and replace them for A.

As used herein, the term "validate" or "validating" a target within a certain system such as the respiratory, inflammatory, CNS, cardiopulmonary, renal immune, and other systems, refers to a process which starts by selecting a therapeutic area within the system, for example, in the CNS or the cardiovascular or cardiopulmonary systems, among others. Areas that may be selected for study within those systems are those of anxiety, mood disorders, satiety and regulation of appetite, pain, cognition, sleep induction and disorders, regulation of temperature, and many others that are controlled by or regulated through the brain, and others exemplified in Table 2 below.

The next step is to select a gene sequence data base corresponding to the appropriate system, e.g. a CNS, lung, cardiac system, kidney/renal system, blood, immune system, pulmonary and respiratory system, sexual function/dysfunction, skin, eye, nasal passages, scalp, testes, cervix, oral cavity, pharynx, esophagus, small and large intestine, synvial tissues, ovaries, ear canal, and other systems. Target genes are selected amongst those known to be associated with the CNS, or with a certain area of the CNS, and those of unknown functions. Genes whose functions are known and might occur in the system may also be selected. Anti-sense oligos are then designed for these target genes or mRNA fragments as described here. Finally, oligos may be selected for their ability to ablate or significantly reduce the expression of the target gene by in vitro hybridization to cell and tissue DNA and RNA. Oligos that show high in vitro down regulation, ablation or expression inhibition are then applied in in vivo tests, preferably by site specific administration, e.g. to a region of the brain, heart, kidney, lung, etc., and pre-determined behavioral, biophysical, biochemical, cognitive, motor, sensory, physiological, and other functions, assessed to establish whether or not a correlation exists between the target and the function. In the respiratory system, for example, a correlation may be shown be decreasing the likelihood that the subject administered such treatment will manifest symptoms of a respiratory or inflammatory lung disease or other lung conditions, such as a malignancy. As applied here, the term "down-regulate" refers to inducing a decrease in production, secretion or availability (and thus a decrease in concentration) of the targeted intracellular protein, which may include a complete ablation.

Table 2: Examples of Diseases & Conditions Associated with Targets & Networks

Dementia	Stroke	Anxiety Antinociception	Analgesia
Cardiopulm. Funct.	Behavioral	Traumatic	Organic Brain
Autonomic Control	Disorders	Brain Injury	Disease
Degen. Encephalopathy	Developmental Drug Sensitivity		
CNS Disord. (Viral, etc.)	Abnorm.&Deform. (Legal & Illegal)		Vision
Cognition	Satiety Food	Alcohol Sensitivity	Heart Attacks
Learning	Depression	Ingestion	Brain Inflammation
Anesthesia	Hearing Olfaction	Hypoxia	Schizophrenia
Brain Cancer	Cranial Def. Memory	Neuropathy	Neurogenic Pain
Dental Pain	Headache	Sensation	Motor Coord.
Mood disorders	Mood Elevat.	Bipolar Dis.	Eating Dis.
Cachexia	AneurismsCardiac & Vasc. Congestive Cardiac & Vasc.		
Cardiac & Vasc. Exudation		Heart Disease	Pain
Inflammation	Stroke	Angina	Heart Failure
Ischemia	Plaque FormationRestenosis		Viral Infec.
Arrythmias	Vascular Permeab.	Arterial Degener.	Libido
Angiogen. & Inhib.	Structural & Biochem. Defects Anger		Transplant. Reject.

The present invention is concerned primarily with target validation in vertebrates, and within this group, of mammals, including human and non-human simians, wild and domesticated animals, marine and land animals, household pets, and zoo animals, for example, felines, canines, equines, pachiderms, cetaceans, and still more preferably to human subjects. One particularly suitable application of this technology is for veterinary purposes, and includes all types of small and large animals in the care of a veterinarian, including wild animals, marine animals, household animals, zoo animals, and the like. Targeted genes and proteins are preferably mammalian, and the sequences targeted are preferably of the same species as the subject being treated. Although in many instances, targets of a different species are also suitable, particularly those segments of the target RNA or gene that display greater than about 45% homology, preferably greater than about 85% homology, still more preferably greater than about 95% homology, with the recipient's sequence. A preferable group of agents is composed of desA anti-sense oligos. Another preferred group is composed of non-fully desA oligonucleotides, where one or more adenosine bases are replaced with universal or alternative bases.

The terms "anti-sense" oligonucleotides generally refers to small, synthetic oligonucleotides, resembling single-stranded DNA, which in this patent are applied to the inhibition of gene expression by inhibition of a target messenger RNA (mRNA). See, Milligan, J. F. et al., J. Med. Chem. 36(14), 1923-1937 (1993), the relevant portion of which is hereby incorporated in its entirety by reference. The present method utilizes anti-sense agents to inhibit gene expression of target genes, including those listed in Table 1 above. This is generally attained by hybridization of the anti-sense oligonucleotides to coding (sense) sequences of a targeted messenger RNA (mRNA), as is known in the art. The exogenously administered agents of the invention decrease the levels of mRNA and protein encoded by the target gene and/or cause changes in the growth characteristics or shapes of the thus treated cells. See, Milligan et al. (1993); Helene, C. and Toulme, J. Biochim. Biophys. Acta 1049, 99-125 (1990); Cohen, J. S. D., Ed., Oligodeoxynucleotides as Anti-sense Inhibitors of Gene Expression; CRC Press: Boca Raton, FL (1987), the relevant portion of which is hereby incorporated in its entirety by reference. As used herein, "anti-sense oligonucleotide" is generally a short

sequence of synthetic nucleotide that (1) hybridizes to any segment of a mRNA encoding a targeted protein under appropriate hybridization conditions, and which (2) upon hybridization causes a decrease in gene expression of the targeted protein. The terms "desAdenosine" (desA) and "des-thymidine" (desT) refer to oligonucleotides substantially lacking either adenosine (desA) or thymidine (desT). In some instances, the des T sequences are naturally occurring, and in others they may result from substitution of an undesirable nucleotide (A) by another one lacking its undesirable activity. In the present context, the substitution is generally accomplished by substitution of A with a "universal or alternative base", as is known in the art.

The mRNA sequence of the targeted protein may be derived from the nucleotide sequence of the gene expressing the protein, whether for existing targets or those to be found in the future. Sequences for many target genes of different systems are presently known. See, GenBank data base, NIH, the entire sequences of which are incorporated here by reference. The sequences of those genes, whose sequences are not yet available, may be obtained by isolating the target segments applying technology known in the art. Once the sequence of the gene, its RNA and/or the protein are known, anti-sense oligonucleotides are produced as described above and utilized to validate the target by in vivo administration and testing for a reduction of the production of the targeted protein in accordance with standard techniques, and of specific functions. In one aspect of this invention, the anti-sense oligonucleotide has a sequence which specifically binds to a portion or segment of an mRNA molecule which encodes a protein associated with a disease or condition of a specific system, e.g. CNS, respiratory, pulmonary, motor, sensory, hormone regulatory, cardiac, renal, immune, blood, cancer genes, and the like. One effect of this binding is to reduce or even prevent the translation of the corresponding mRNA and, thereby, reduce the available amount of target protein in the subject's lung.

In one preferred embodiment of this invention, one or more of the phosphodiester residues of the anti-sense oligonucleotide are modified or substituted. Chemical analogs of oligonucleotides with modified or substituted phosphodiester residues, e.g., to the methylphosphonate, the phosphotriester, the phosphorothioate, the phosphorodithioate, or the phosphoramidate, which increase the in vivo stability of the oligonucleotide are particularly preferred. The naturally occurring phosphodiester linkages of oligonucleotides are susceptible to some degree of degradation by cellular nucleases. Many of the residues proposed herein, on the contrary, are highly resistant to nuclease degradation. See Milligan et al., and Cohen, J. S. D., supra. In another preferred embodiment of the invention, the oligonucleotides may be protected from degradation by adding a "3'-end cap" by which nuclease-resistant linkages are substituted for phosphodiester linkages at the 3' end of the oligonucleotide. See, Tidd, D. M. and Warenius, H.M., Be. J. Cancer 60: 343-350 (1989); Shaw, J.P. et al., Nucleic Acids Res. 19: 747-750 (1991), the relevant section of which are incorporated in their entireties herein by reference. Phosphoramidates, phosphorothioates, and methylphosphonate linkages all function adequately in this manner for the purposes of this invention. The more extensive the modification of the phosphodiester backbone the more stable the resulting agent, and in many instances the higher their RNA affinity and cellular permeation. See Milligan, et al., supra. Thus, the number of residues which may be modified or substituted will vary depending on the need, target, and route of administration, and may be from 1 to all the residues, to any number in between. Many different methods for replacing the entire phosphodiester backbone with novel linkages are known. See, Millikan et al, supra. Preferred analogue residues for the base, the internucleotide linkage, or the sugar include phosphorothioate, methylphosphonate, phosphotriester, thioformacetal, phosphorodithioate, phosphoramidate, formacetal boranophosphate, 3'-thioformacetal, 5'-thioether, carbonate, 5'-N-carbamate, sulfate, sulfonate, sulfamate, sulfonamide, sulfone, sulfite., 2'-O methyl, sulfoxide, sulfide, hydroxylamine, methoxy methyl (MOM), methoxy ethyl (MOE), methylene(methylimino) (MMI), and methyleneoxy(methylimino) (MOMI) residues. Phosphorothioate and

methylphosphonate-modified oligonucleotides are particularly preferred due to their availability through automated oligonucleotide synthesis. See, Millikan et al, supra. Where appropriate, the agent of this invention may be administered in the form of a pharmaceutically acceptable salt, or as a mixture of the anti-sense oligonucleotide and a salt. In another embodiment of this invention, a mixture of different anti-sense oligonucleotides or their pharmaceutically acceptable slats is administered. The agents of this invention have the capacity to attenuate the expression of one target mRNA and/or to enhance or attenuate the activity of one pathway. By means of example, the present method may be practiced by identifying all possible deoxyribonucleotide segments which are low in thymidine (T) or deoxynucleotide segments low in adenosine (A) of about 7 or more mononucleotides, preferably up to about 60 mononucleotides, more preferably about 10 to about 36 mononucleotides, and still more preferably about 12 to about 21 mononucleotides, in a target mRNA or a gene, respectively. This may be attained by searching for mononucleotide segments within a target sequence which are low in, or lack thymidine (RNA), a nucleotide which is complementary to adenosine, or that are low in adenosine (gene), that are 7 or more nucleotides long. In most cases, this search typically results in about 10 to 30 such sequences, i.e. naturally lacking or having less than about 40% adenosine, anti-sense oligonucleotides of varying lengths for a typical target mRNA of average length, i. e., about 1800 nucleotides long. Those with high content of T or A, respectively, may be fixed by substitution of a universal or alternative base for one or more As.

The agent(s) of this invention may be of any suitable length, including but not limited to, about 7 to about 60 nucleotides long, preferably about 12 to about 45, more preferably up to about 30 nucleotides long, and still more preferably up to about 21, although they may be of other lengths as well, depending on the particular target and the mode of delivery. The agent(s) of the invention may be directed to any and all segments of a target RNA. One preferred group of agent(s) includes those directed to an mRNA region containing a junction between an intron and an exon. Where the agent is directed to an intron/exon junction, it may either entirely overlie the junction or it may be sufficiently close to the junction to inhibit the splicing-out of the intervening exon during processing of precursor mRNA to mature mRNA, e.g. with the 3' or 5' terminus of the anti-sense oligonucleotide being positioned within about, for example, within about 2 to 10, preferably about 3 to 5, nucleotide of the intron/exon junction. Also preferred are anti-sense oligonucleotides which overlap the initiation codon, and those near the 5' and 3' termini of the coding region. The anti-sense oligo may have an adenosine content of about 0, about 3%, about 5%, about 7% to about 8%, about 10%, about 12%, about 15%, and any intermediate amounts and ranges of adenosine content. In the present method, one or more or all A may be substituted by a universal or alternative base such as heteroaromatic bases which bind to thymidine but have less than about 0.5, about 0.3, about 0.1 of the agonist or antagonist activity of adenosine at the adenosine A_1, A_{2a}, A_{2b} and A_3 receptors, and heteroaromatic bases which have no activity at the adenosine A_{2a} receptor. The alternative base may be 3-nitropyrrole-2'-deoxynucleoside, 5-nitro-indole, 2-deoxyribosyl-(5-nitroindole), 2-deoxyribofuranosyl-(5-nitroindole), 2'-deoxyinosine, 2'-deoxynebularine, 6H, 8H-3,4-dihydropyrimido [4,5-c] oxazine-7-one or 2-amino-6-methoxyaminopurine, among others. The oligo may have a methylated cytosine (^{m}C) substituted for one or more, or all unmethylated C in a CpG dinucleotide (s), if the latter is (are) present in the oligo(s). Other C_5 modifications to pyrimidines are also embodiments of the current invention, e.g. C_5 propyne, etc. One or more, or all nucleotide linking residues of the oligos suitable for use with the present method may be methylphosphonate, phosphotriester, phosphorothioate, phosphorodithioate, boranophosphate, formacetal, thioformacetal, thioether, carbonate, carbamate, sulfate, sulfonate, sulfamate, sulfonamide, sulfone, sulfite, sulfoxide, sulfide, hydroxylamine, methylene(methyimino), (MMI), methoxymethyl (MOM), methoxyethyl (MOE), methyleneoxy (methylimino) (MOMA), methoxy methyl (MOM), 2'-O-methyl, phosphoramidate, and C-5 substituted (C-5

propyne) residues and combinations thereof. The oligo used in the present method may be linked to a vector, such as a prokaryotic or eukaryotic vector, many of which are known in the art. The method of the invention prescribes the administration of an amount of anti-sense oligo effective to reduce the production or availability, or to increase the degradation, of the mRNA, or to reduce the amount of the polypeptide present in the lungs. The administration is preferably done in situ, e.g. directly into the respiratory system or nasal passage, e.g. by inhalation or applied to the subject's lungs, for respiratory and pulmonary targets. The anti-sense oligo may be administered directly into the brain, heart, kidney, tumor, testes, eyes, ear passage, cervix, nasal passage, scalp, oral cavity, muscle, pharynx, esophagus, intestines, rectum, synovial tissue, ovaries, and other localized tissues by injection, by stereotactic insertion, or in vitro, among other methods. In addition, the oligo may also be administered into the blood when the target is part and parcel of the circulatory and immune systems. In the latter case, wherein the disease or condition is associated with an immunological dysfunction, the target may be immunoglobulins, antibody receptors, cytokines, cytokine receptors, gene(s) and the corresponding mRNA(s) encoding them, the genes and mRNA flanking regions and intron and exon borders, among others. Wherein the disease or condition is associated with a malignancy or cancer, the target may be selected from growth regulation associated enzyme and other proteins, immunoglobulins and antibody receptors, gene(s) and mRNA(s) encoding them, genes and mRNAs associated with oncogenes, and genomic and mRNA flanking regions and exon and intron borders. Additionally, certain genes of normal cells that are involved in the cancer process, such as angiogenesis factors, adhesion molecules and protease enzymes involved in metastases and others are also part of the invention. The method may be practiced, for example, by administering the composition in vitro, orally, intracavitarily, intranasally, intraanally, intravaginally, intrauterally, intracranially, pulmonarily, intrarenally, intranodularly, intraarticularly, intraotically, intralymphatically, transdermally, intrabucally, intravenously, subcutaneously, intramuscularly, intratumorously, intraglandularly, intraocularly, intracranial, into an organ, intravascularly, intrathecally, by implantation, by inhalation, intradermally, intrapulmonarily, into the ear, into the heart, by slow release, by sustained release and by a pump. Other examples of targets are genes and mRNAs encoding polypeptides selected from the group consisting of transcription factors, stimulating and activating factors, cytokines and their receptors, interleukins, interleukin receptors, chemokines, chemokine receptors, endogenously produced specific and non-specific enzymes, immunoglobulins, antibody receptors, central nervous system (CNS) and peripheral nervous and non-nervous system receptors, CNS and peripheral nervous and non-nervous system peptide transmitters, adhesion molecules, defensines, growth factors, vasoactive peptides, peptide receptors and binding protein, and genes and mRNAs corresponding to oncogenes, G-protein coupled receptors, etc. The anti-sense oligo(s) may be produced by selection of a target from polypeptides associated with diseases and conditions afflicting lung airways, such as difficult respiratory activity and malignancies, increased or decreased surfactant secretion, and many others, genes and RNAs encoding them, the genomic and mRNA flanking regions and the gene(s) and mRNA(s) intron and exon borders; obtaining the sequence of a mRNA(s) selected from the group consisting of mRNAs corresponding to the target gene(s) and mRNAs encoding the target polypeptide(s), genomic and mRNA flanking regions and the genes and mRNAs intron and exon borders; selecting at least one segment of the mRNA(s); synthesizing one or more oligo anti-sense to the selected mRNA segment(s); and substituting, if necessary, an alternative base(s) capable of hybridizing to thymidine (T) but having reduce or no agonist capacity at the adenosine receptors (no or reduced adenosine receptor activation) for one or more A(s) to reduce the content of A present in the oligo to up to about 15% of all nucleotides. As already indicated, suitable targets are target proteins, genes and mRNAs encoding polypeptides selected from transcription factors, stimulating and activating factors, interleukins, interleukin receptors, chemokines, chemokine receptors, endogenously produced specific and non-specific enzymes,

immunoglobulins, antibody receptors, central nervous system (CNS) and peripheral nervous and non-nervous system receptors, CNS and peripheral nervous and non-nervous system peptide transmitters and their receptors, adhesion molecules, defensines, growth factors, vasoactive peptides and their receptors, and binding proteins, and target genes and mRNAs corresponding to oncogenes, and their flanking regions and intron and exon borders. The encoded polypeptides may be selected from NfκB Transcription Factor, Interleukin-8 Receptor (IL-8 R), Interleukin 5 Receptor (IL-5 R), Interleukin 4 Receptor (IL-4 R), Interleukin 3 Receptor (IL-3 R), Interleukin-1β (IL-1β), Interleukin 1β Receptor (IL- 1β R), Eotaxin, Tryptase, Major Basic Protein, β2-adrenergic Receptor Kinase, Endothelin Receptor A, Endothelin Receptor B, Preproendothelin, Bradykinin B2 Receptor, IgE High Affinity Receptor, Interleukin 1 (IL-1), Interleukin 1 Receptor (IL-1 R), Interleukin 9 (IL-9), Interleukin-9 Receptor (IL-9 R), Interleukin 11 (IL-11), Interleukin-11 Receptor (IL-11 R), Inducible Nitric Oxide Synthase, Cyclooxygenase (COX), Intracellular Adhesion Molecule 1 (ICAM-1) Vascular Cellular Adhesion Molecule (VCAM), Rantes, Endothelial Leukocyte Adhesion Molecule (ELAM-1), Monocyte Activating Factor, Neutrophil Chemotactic Factor, Neutrophil Elastase, Defensin 1, 2 and 3, Muscarinic Acetylcholine Receptors, Platelet Activating Factor, Tumor Necrosis Factor α, 5-lipoxygenase, Phosphodiesterase IV, Substance P, Substance P Receptor, Histamine Receptor, Chymase, CCR-1 CC Chemokine Receptor, CCR-2 CC Chemokine Receptor, CCR-3 CC Chemokine Receptor, CCR-4 CC Chemokine Receptor, CCR-5 CC Chemokine Receptor, Prostanoid Receptors, GATA-3 Transcription Factor, Neutrophil Adherence Receptor, MAP Kinase, Interleukin-9 (IL-9), NFAT Transcription Factors, STAT 4, MIP-1α, MCP-2, MCP-3, MCP-4, Cyclophillins, Phospholipase A2, Basic Fibroblast Growth Factor, Metalloproteinase, CSBP/p38 MAP Kinase, Tryptose Receptor, PDG2, Interleukin-3 (IL-3), Interleukin-1β (IL-1β), Cyclosporin A-Binding Protein, FK5-Binding Protein, α4β1 Selectin, Fibronectin, α4β7 Selectin, Mad CAM-1, LFA-1 (CD11a/CD18), PECAM-1, LFA-1 Selectin, C3bi, PSGL-1, E-Selectin, P-Selectin, CD-34, L-Selectin, p150,95, Mac-1 (CD11b/CD18), Fucosyl transferase, VLA-4, CD-18/CD11a, CD11b/CD18, ICAM2 and ICAM3, C5a, CCR3 (Eotaxin Receptor), CCR1, CCR2, CCR4, CCR5, LTB-4, AP-1 Transcription Factor, Protein kinase C, Cysteinyl Leukotriene Receptor, Tachychinnen Receptors (tach R), IκB Kinase 1 & 2, STAT 6, c-mas and NF-Interleukin-6 (NF-IL-6), and their flanking regions and intron and exon borders. However, this invention is primarily intended for application to newly discovered genes not yet available in public data bases. It is for this group of genes that target validation is of most importance, for without it their role in disease remains unknown.

Table 3 below provides a short list of targets to which the agents of the invention are effectively applied. These are by way of example only. The method is applicable to any system in which target validation would be discussed by virtue of bioactive adenosine release during oligonucleotie degradation. This is of importance because adenosine is anti-inflammatory and its release would obscure cancer target validation results.

Table 3: Cancer Targets

Transforming Oncogenes	Therapy Targets
ras	Thymidylate Synthetase
src	Thymidylate Synthetase
myc	Dihydrofolate Reductase
bcl-2	Thymidine Kinase
	Deoxycytidine Kinase
	Ribonucleotide Reductase
Angiogenesis factors	Adhesion Molecules
Oncogenes	Folate Pathway Enzymes
DNA repair genes	(One Carbon Pool)
	Telomerase
	HMG CoA Reductase
	Farnesyl Transferase
	Glucose-6-Phosphate Transferase

A group of preferred targets for the validation of cancer targets are genes associated with different types of cancers, or those generally known to be associated with malignancies, whether they are regulatory or involved in the production of RNA and/or proteins. Examples are transforming oncogenes, targets which are shown, among others, in Table 3 above. Other targets which present cancer target validation agents are directed to are various enzymes, primarily, although not exclusively, thymidylate synthetase, dihydrofolate reductase, thymidine kinase, deoxycytidine kinase, ribonucleotide reductase, other gene products more abundantly manufactured in cancer cells than in normal cells, and the like. The present technology is particularly useful in the validation/invalidation of cancer target genes given that traditional cancer therapies are not effective in selectively killing cancer cells while preserving normal living cells from the devastating effects of treatments such as chemotherapy, radiotherapy, and the like. That is, present cancer treatments cannot be selectively targeted to malignant cells. Any target validated by the present method will provide the ability of selectively attenuating a desired gene product and attenuating or enhancing function, and its pathway. This approach provides a significant advantage over standard cancer treatments because it permits the selection of a system, and within the system a pathway including multiple targets, e.g. primary, secondary and possibly tertiary targets, which may not be generally expressed simultaneously in normal cells and validate them separately and jointly. Thus, the present method will provide targets for therapy. Once a target is validated by the present method, a selective agent acting on the target may be administered to a subject to cause a selective increase in toxicity within tumor cells that, for instance, express three targets while normal cells that may expresses only one or two of the targets will be significantly less affected or even spared. The present method administers agents which are preferably designed to be anti-sense to target genes and/or mRNAs related in origin to the species to which it is to be administered. When for validating targets in humans, the agents are preferably designed to be anti-sense to a human gene or RNA. The agents of the invention encompass oligonucleotides which are anti-sense to naturally occurring DNA and/or RNA sequences, fragments thereof of up to a length of one (1) base less than the targeted sequence, preferably at least about 7 nucleotides long, oligos having only over about 0.1%, about 1%, about 4% up to about 5%, about 10%, about 15%, about 30%, or lacking adenosine altogether, and oligos in which one or more of the adenosine nucleotides have been replaced with so-called universal or alternative bases, which may pair up with thymidine nucleotides but fail to substantially trigger adenosine receptor activity. Examples of human

sequences and fragments, which are not limiting, of anti-sense oligonucleotide of the invention are the following fragments as well as shorter segments of the fragments and of the full gene or mRNA coding sequences, exons and intron-exon junctions encompassing preferably 7, 10, 15, 18 to 21, 24, 27, 30, n-1 nucleotides for each sequence, where n is the sequence's total number of nucleotides. These fragments may be selected from any portion of the longer oligo, for example, from the middle, 5'- end, 3'- end or starting at any other site of the original sequence. Of particular importance are fragments of low adenosine nucleotide content, that is, those fragments containing less than or about 30%, preferably less than or about 15%, more preferably less than or about 10%, and even more preferably less than or about 5%, and most preferably those devoid of adenosine nucleotide, either by choice or by replacement with a universal or alternative base in accordance with this invention. The agent of the invention includes as a most preferred group sequences and their fragments where one or more adenosines present in the sequence have been replaced by a universal or alternative base (B), as exemplified here. Similarly, also encompassed are all shorter fragments of the B-containing fragments designed by substitution of B(s) for adenosine(s) (A(s)) contained in the sequences, fragments thereof or segments thereof, as described above.

The present method may utilize the agents by themselves or in the form of pharmaceutical compositions comprising an amount of the anti-sense oligonucleotide as given above effective to reduce the expression of a target protein. The anti-sense oligo must first pass through a cell membrane to bind specifically with mRNA encoding the protein in the cell and prevent its translation. Such compositions are provided in a suitable pharmaceutically acceptable carrier, e.g. sterile pyrogen-free saline solution. The agent of the invention may be formulated with a hydrophobic carrier capable of passing through a cell membrane, e.g. in a liposome, with the liposomes carried in a pharmaceutically acceptable aqueous carrier, optionally and alternatively with surfactant or lipid. In addition, the oligonucleotides may be coupled to an agent which inactivates mRNA, such as a ribozyme to attain a more complete inhibition of translation. The pharmaceutical formulation may also comprise chimeric molecules where the anti-sense oligos are attached to molecules which are known to be internalized by cells. These oligonucleotide conjugates utilize cellular up-take pathways to increase the intracellular concentrations of the oligonucleotide. Examples of molecules used in this manner are macromolecules including transferrin, asialoglycoprotein (bound to oligonucleotides via polylysine) and streptavidin, among others known in the art. The present method may also utilize anti-sense compounds in a pharmaceutical formulation, e.g. within a lipid particle or vesicle, such as a liposome or microcrystal. The particles may be of any suitable structure, such as unilamellar or plurilamellar. The one preferred embodiment, the anti-sense oligonucleotide is comprised within the liposome. Positively charged lipids such as N-[1-(2, 3 -dioleoyloxi) propyl] -N, N, N-trimethylammoniumethylsulfate, or "DOTAP," are particularly preferred for such particles and vesicles. However, others are also suitable and may in fact be more suitable. The preparation of such lipid particles is well known. See, e.g., US Patent Nos. 4,880,635 to Janoff et al., 4,906,477 to Kurono et al., 4,911,928 to Wallach, 4,917,951 to Wallach, 4,920,016 to Allen et al., 4,921,757 to Wheatley et al., the relevant sections of all of which are herein incorporated in their entireties by reference. The method of the invention provides for the administration of the anti-sense oligos by any means, preferably those which afford the least transport, e.g. in situ in the brain, lungs, kidneys, heart, testes, etc.

The administration of the agent(s) to the lungs may be done by any suitable means, but preferably through the respiratory system as a respirable formulation, more preferably in the form of an aerosol comprising respirable particles which, in turn, comprise the agent for respiration or inhalation by the subject. The respirable particles may be in gaseous, liquid or solid form, and they may, optionally, contain other therapeutic ingredients and formulation components. The particles of the present invention are preferably

particles of respirable size, preferably of a size sufficiently small to pass, upon inhalation, through the mouth and larynx and into the bronchi and alveoli of the lungs. In general, particles ranging from about 0.5 to 10 microns in diameter are respirable. However, other sizes may also be suitable. Particles of non-respirable size, of considerably larger diameter, which are included in the respirable formulation tend to deposit in the throat and may be swallowed. Accordingly, it is desirable to minimize the quantity of non-respirable particles in the aerosol. For nasal administration, a particle size in the range of 10-500 :m is preferred to ensure their retention in the nasal cavity. Aerosols of liquid particles comprising the agent may be produced by any suitable means, such as with as insufflator or nebulizer. See, e.g., US Patent No. 4,501,729. Suitable propellants include solvents such as certain chlorofluorocarbon compounds, for example, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane and/or mixtures thereof. Other propellants are suitable and may be preferable when better suited for particular applications. The formulation may additionally comprise one or more co-solvents, for example, ethanol, surfactants, such as oleic acid or sorbitan trioleate, antioxidants and suitable flavoring agents. The anti-sense oligos may be administered to the brain by stereotoxic procedures or by injection to target isolated areas of the CNS, all methods known in the art. Alternatively, the oligos may be administered as a formulation that will cross the blood-brain barrier, as is known in the art, e. g. conjugates of streptavidin and a monoclonal antibody directed to the transferrin receptor may be employed as a universal carrier for the delivery of mono-biotinylated peptides, anti-sense oligos (3'-biotinylation of phosphodiesters or other derivatives) and peptide-oligos to the brain. See, for example, Levy, R.M. et al., J. Neuroviral 3 Suppl: 574-75 (1997); Wu-Pong and Gewirtz, BioPharm, pp. 32-38 (Jan. 1999); Boado, R. J., et al., J. Pharm. Sci. 87 (11): 1308-1315 (1998). The administration to the heart, liver and kidneys as well as other organs, may be conducted by in situ administration techniques such as catheterization, injection, and regional diffusion, all of which are known in the art. See, for example, Lewis, K.J. et al., J. Drug Target, 5(4): 291 (1998); Ayrin, M.A. et al., Cathet. Cardiovasc. Diagn, 41(3): 232-240 (1997); Luft, F.C., J. Molec. Med. 76(2): 75 (1998). These administrations are typically conducted with liquid, solid or gaseous pharmaceutical compositions of the agent, that may be prepared by combining the anti-sense oligo with a suitable vehicle or carrier, such as sterile pyrogen-free water, lipid, and/or other known pharmaceutically or veterinarily acceptable carrier. Other therapeutic compounds may be included as well as other formulation ingredients as is known in the art. Solid particulate compositions comprising dry particles of, e.g. the micronized agent of the invention may be prepared by grinding the dry anti-sense compound with a mortar and pestle, and then passing the thus ground, e.g. micronized composition through a screen, e.g. 400 mesh screen, to break up or separate large agglomerates of particles. A solid particulate composition comprising the anti-sense compound may optionally also comprise a dispersant and other known agents, which serve to facilitate the formation of a mist or aerosol. A suitable dispersant is lactose, which may be blended with the anti-sense compound in any suitable ratio, about 1:1 w/w. Other ratios may be utilized as well, and other therapeutic and formulation agents may also be included. The relevant sections of the references cited in this patent are intended for incorporation to this text by referene, particularly of those publications and patents which facilitate the enablement and written description of the various aspects of the invention.

The dosage of the anti-sense compound administered generally varies with the target, the function and its amplification, and disease being investigated, the condition of the subject, the particular formulation, the route and site of administration, the timing of administration, etc. In general, it is desirable to attain intracellular concentrations of the oligonucleotide of from 0.05 to 50 :M, or more particularly 0.2 to 5 :M. However, a dose-response curve may suitably be determined to establish a proper dose to observe a clear response. The dosage utilized may be varied, e.g. from about 0.001, about 0.01, about 1 mg/kg to about 50, about 100, and about 150 mg/kg are typically employed. Higher and lower doses may also be administered as

an artisan will see suitable for specific application. These amounts may be administered once or over a period of time, e.g. every 24 hrs where needed, although other regimens are also suitable. The following examples are provided to illustrate the present invention, and should not be construed as limiting thereon. In these examples, :M means micromolar, ml means milliliters, :m means micrometers, mm means millimeters, cm means centimeters, EC means degrees Celsius, :g means micrograms, mg means milligrams, g means grams, kg means kilograms, M means molar, and hrs. means hours.

EXAMPLES

Example 1: Design and Synthesis of Anti-sense Oligonucleotides

The design of anti-sense oligonucleotides against target receptors may require the solution of the complex secondary structure of the target receptor mRNA. After generating this structure, anti-sense nucleotide are designed which target regions of mRNA which might be construed to confer functional activity or stability to the mRNA and which optimally may overlap the initiation codon. Other target sites are readily usable. As a demonstration of specificity of the anti-sense effect, other oligonucleotides not totally complementary to the target mRNA, but containing identical nucleotide compositions on a w/w basis, are included as controls in anti-sense experiments. For example, the mRNA secondary structure of the adenosine A_1 receptor was analyzed and used as described above. to design a phosphorothioate anti-sense oligonucleotide. The anti-sense oligonucleotide which was synthesized was designated $HAdA_1AS$ and had the following sequence: 5' -GAT GGA GGG CGG CAT GGC GGG-3' (**SEQ ID NO:1**). As a control, a mismatched phosphorothioate anti-sense nucleotide designated HAdAlMM1 was synthesized with the following sequence: 5' -GTA GCA GGC GGG GAT GGG GGC-3' (**SEQ ID NO:2**). Each oligonucleotide had identical base content and general sequence structure. Homology searches in GENBANK (release 85.0) and EMBL (release 40.0) indicated that the anti-sense oligonucleotide was specific for the human and rabbit adenosine A_1 receptor genes, and that the mismatched control was not a candidate for hybridization with any known gene sequence. The secondary structure of the adenosine A_3 receptor mRNA was similarly analyzed and used as described above to design two phosphorothioate anti-sense oligonucleotides. The first anti-sense oligonucleotide (HAdA3AS1) synthesized had the following sequence: 5' -GTT GTT GGG CAT CTT GCC-3' (**SEQ ID NO:3**). As a control, a mismatched phosphorothioate anti-sense oligonucleotide (HAdA3MM1) was synthesized, having the following sequence: 5' -GTA CTT GCG GAT CTA GGC-3' (**SEQ ID NO:4**). A second phosphorothioate anti-sense oligonucleotide (HAdA3AS2) was also designed and synthesized, having the following sequence: 5' -GTG GGC CTA GCT CTC GCC-3' (**SEQ ID NO:5**). Its control oligonucleotide (HAdA3MM2) had the sequence: 5' -GTC GGG GTA CCT GTC GGC-3' (**SEQ ID NO:6**). Phosphorothioate oligonucleotides were synthesized on an Applied Biosystems Model 396 Oligonucleotide Synthesizer, and purified using NENSORB chromatography (DuPont, MD).

Example 2: In Vivo Testing of Adenosine A_1 Receptor Anti-sense Oligos

The anti-sense oligonucleotide against the human A_1 receptor (**SEQ ID NO:1**) described above. was tested for efficacy in an in vitro model utilizing lung adenocarcinoma cells HTB-54. HTB-54 lung adenocarcinoma cells were demonstrated to express the A_1 adenosine receptor using standard northern blotting procedures and receptor probes designed and synthesized in the laboratory. HTB-54 human lung adenocarcinoma cells (106/100 mm tissue culture dish) were exposed to 5.0 :M HAdAlAS or HAdAlMM1 for 24 hours, with a fresh change of media and oligonucleotides after 12 hours of incubation. Following 24 hour exposure to the oligonucleotides, cells were harvested and their RNA extracted by standard procedures. A 21-

mer probe corresponding to the region of mRNA targeted by the anti-sense (and therefore having the same sequence as the anti-sense, but not phosphorothioated) was synthesized and used to probe northern blots of RNA prepared from HAdAlAS-treated, HAdAlMM1-treated and non-treated HTB-54 cells. These blots showed clearly that HAdAlAS but not HAdAlMM1 effectively reduced human adenosine receptor mRNA by >50%. This result showed that HAdAlAS is a good candidate for an anti-asthma drug since it depletes intracellular mRNA for the adenosine A_1 receptor, which is involved in asthma.

Example 3: In Vivo Efficacy of Adenosine A_1 Receptor Anti-sense Oligos

A fortuitous homology between the rabbit and human DNA sequences within the adenosine A_1 gene overlapping the initiation codon permitted the use of the phosphorothioate anti-sense oligonucleotides initially designed for use against the human adenosine A_1 receptor in a rabbit model. Neonatal New Zealand white Pasteurella-free rabbits were immunized intraperitoneally within 24 hours of birth with 312 antigen units/ml house dustmite (D. farinae) extract (Berkeley Biologicals, Berkeley, CA), mixed with 10% kaolin. Immunizations were repeated weekly for the first month and then biweekly for the next 2 months. At 3-4 months of age, eight sensitized rabbits were anesthetized and relaxed with a mixture of ketamine hydrochloride (44 mg/kg) and acepromazine maleate (0.4 mg/kg) administered intramuscularly. The rabbits were then laid supine in a comfortable position on a small molded, padded animal board and intubated with a 4.0-mm intratracheal tube (Mallinkrodt, Inc., Glens Falls, NY). A polyethylene catheter of external diameter 2.4 mm with an attached latex balloon was passed into the esophagus and maintained at the same distance (approximately 16 cm) from the mouth throughout the experiments. The intratracheal tube was attached to a heated Fleisch pneumotachograph (size 00; DOM Medical, Richmond, VA), and flow was measured using a Validyne differential pressure transducer (Model DP-45161927; Validyne Engineering Corp., Northridge, CA) driven by a Gould carrier amplifier (Model 11-4113; Gould Electronic, Cleveland, OH). The esophageal balloon was attached to one side of the differential pressure transducer, and the outflow of the intratracheal tube was connected to the opposite side of the pressure transducer to allow recording of transpulmonary pressure. Flow was integrated to give a continuous tidal volume, and measurements of total lung resistance (RL) and dynamic compliance (Cdyn) were calculated at isovolumetric and flow zero points, respectively, using an automated respiratory analyzer (Model 6; Buxco, Sharon, CT). Animals were randomized and on Day 1 pretreatment values for PC50 were obtained for aerosolized adenosine. Anti-sense (HAdAlAS) or mismatched control (HAdAlMM) oligonucleotides were dissolved in sterile physiological saline at a concentration of 5000 :g (5 mg) per 1.0 ml. Animals were subsequently administered the aerosolized anti-sense or mismatch oligonucleotide via the intratracheal tube (approximately 5000 :g in a volume of 1.0 ml), twice daily for two days. Aerosols of either saline, adenosine, or anti-sense or mismatch oligonucleotides were generated by an ultrasonic nebulizer (DeVilbiss, Somerset, PA), producing aerosol droplets 80% of which were smaller than 5 :m in diameter. In the first arm of the experiment, four randomly selected allergic rabbits were administered anti-sense oligonucleotide and four the mismatched control oligonucleotide. On the morning of the third day, PC50 values (the concentration of aerosolized adenosine in mg/ml required to reduce the dynamic compliance of the bronchial airway 50% from the baseline value) were obtained and compared to PC50 values obtained for these animals prior to exposure to oligonucleotide. Following a 1 week interval, animals were crossed over, with those previously administered mismatch control oligonucleotide now administered anti-sense oligonucleotide, and those previously treated with anti-sense oligonucleotide now administered mismatch control oligonucleotide. Treatment methods and measurements were identical to those employed in the first arm of the experiment. It should be noted that in six of the eight animals treated with anti-sense oligonucleotide, adenosine-mediated bronchoconstriction could not be obtained up to the limit of

solubility of adenosine, 20 mg/ml. For the purpose of calculation, PC50 values for these animals were set at 20 mg/ml. The values given therefore represent a minimum figure for anti-sense effectiveness. Actual effectiveness was higher. The results of this experiment are illustrated in Table 4 below.

Table 4: **Effect of Adenosine A_1 Receptor Anti-sense Oligo upon PC50 Values in Asthmatic Rabbits**

Mismatch Control		A_1 Receptor Anti-sense Oligo	
Pre Oligonucleotide	**Post Oligonucleotide**	**Pre Oligonucleotide**	**Post Oligonucleotide**
3.56 ± 1.02	5.16 ± 1.03	2.36 ± 0.68	>19.5 ± 0.34**

The results are presented as the mean (n=8) ± SEM.
The significance was determined by repeated-measures analysis of variance (ANOVA), and Tukey's protected test.
**Significantly different from all other groups, p<0.01.

In both arms of the experiment, animals receiving the anti-sense oligonucleotide showed an order of magnitude increase in the dose of aerosolized adenosine required to reduce dynamic compliance of the lung by 50%. No effect of the mismatched control oligonucleotide upon PC50 values was observed. No toxicity was observed in any animal receiving either anti-sense or control inhaled oligonucleotide. These results show clearly that the lung has exceptional potential as a target for anti-sense oligonucleotide-based therapeutic intervention in lung disease. They further show, in a model system which closely resembles human asthma, that down regulation of the adenosine A_1 receptor largely eliminates adenosine-mediated bronchoconstriction in asthmatic airways. Bronchial hyperresponsiveness in the allergic rabbit model of human asthma is an excellent endpoint for anti-sense intervention since the tissues involved in this response lie near to the point of contact with aerosolized oligonucleotides, and the model closely simulates an important human disease.

Example 4: **Specificity of A_1-adenosine Receptor Anti-sense Oligonucleotide**

At the conclusion of the cross-over experiment of Example 3 above, airway smooth muscle from all rabbits was quantitatively analyzed for adenosine A_1 receptor number. As a control for the specificity of the anti-sense oligonucleotide, adenosine A_2 receptors, which should not have been affected, were also quantified. Airway smooth muscle tissue was dissected from each rabbit and a membrane fraction prepared according to the method of Kleinstein et al. (Kleinstein, J. and Glossmann, H., Naunyn-Schmiedeberg's Arch. Pharmacol. 305: 191-200 (1978)), the relevant portion of which is hereby incorporated in its entirety by reference, with slight modifications. Crude plasma membrane preparations were stored at 70EC until the time of assay. Protein content was determined by the method of Bradford (M. Bradford, Anal. Biochem. 72, 240-254 (1976), the relevant portion of which is hereby incorporated in its entirety by reference). Frozen plasma membranes were thawed at room temperature and were incubated with 0.2 U/ml adenosine deaminase for 30 minutes at 37EC to remove endogenous adenosine. The binding of [^{3}H] DPCPX (A_1 receptor-specific) or [^{3}H] CGS-21680 (A_1 receptor-specific) was measured as previously described by Ali et al. (Ali, S. et al., J. Pharmacol. Exp. Ther. 268, Am. J. Physiol 266, L271-277 (1994), the relevant portion of which is hereby incorporated in its entirety by reference). The animals treated with adenosine A_1 anti-sense oligonucleotide in the cross-over experiment had a nearly 75% decrease in A_1 receptor number compared to controls, as assayed by specific binding of the A_1-specific antagonist DPCPX. There was no change in adenosine A_2 receptor number, as assayed by specific binding of the A_2 receptor-specific agonist 2- [p- (2-carboxyethyl)-phenethylamino] -5' -

(N-ethylcarboxamido) adenosine (CGS-21680). This is illustrated in Table 5 below. The results below illustrate the effectiveness of anti-sense oligonucleotides in treating airway disease. Since the anti-sense oligos described above eliminate the receptor systems responsible for adenosine-mediated bronchoconstriction, it may be less imperative to eliminate adenosine from them. However, it would be preferable to eliminate adenosine from even these oligonucleotides to reduce the dose needed to attain a similar effect. Described above are other anti-sense oligonucleotides targeting mRNA of proteins involved in inflammation. Adenosine has been eliminated from their nucleotide content to prevent its liberation during degradation.

Table 5: **Specificity of Action of Adenosine A_1 Receptor Anti-sense Oligonucleotide**

	Mismatch Control Oligonucleotide	A_1 Anti-sense Oligonucleotide
A_1-Specific Binding	1105 ± 48**	293 ± 18
A_2-Specific Binding	302 ± 22	442 ± 171

The results are presented as the mean (n = 8) ± SEM.
The significance was determined by repeated-measures analysis of variance (ANOVA), and Tukey's protected test.
**Significantly different from mismatch control, $p<0.01$.

Example 5: **Anti-sense Oligos Directed to Other Target Nucleic Acids**

This work was conducted to demonstrate that the present invention is broadly applicable to anti-sense oligonucleotides ("oligos") specific to nucleic acid targets broadly. The following experimental studies were conducted to show that the method of the invention is broadly suitable for use with anti-sense oligos designed as taught by this application and targeted to any and all adenosine receptor mRNAs. For this purpose, various anti-sense oligos were prepared to adenosine receptor mRNAs exemplified by the adenosine A_1, A_{2b} and A_3 receptor mRNAs. Anti-sense Oligo I was disclosed above (**SEQ ID NO: 1**).Five additional anti-sense phosphorothioate oligos were designed and synthesized as indicated above.

1- Oligo II (**SEQ ID NO: 7**) also targeted to the adenosine A_1 receptor, but to a different region than Oligo I.
2- Oligo V (**SEQ ID NO: 10**) targeted to the adenosine A_{2b} receptor.
3- Oligos III (**SEQ ID NO: 8**) and IV (**SEQ ID NO: 9**) targeted to different regions of the adenosine A_3 receptor.
4- Oligo I-PD (**SEQ ID NO: 11**)(a phosphodiester oligo of the same sequence as Oligo I).

These anti-sense oligos were designed for therapy on a selected species as described above and are generally specific for that species, unless the segment of the target mRNA of other species happens to contain a similar sequences. All anti-sense oligos were prepared as described below, and tested in vivo in a rabbit model for bronchoconstriction, inflammation and allergy, which have breathing difficulties and impeded lung airways, as is the case in ailments such as asthma, as described in the above-identified application.

Example 6: **Design & Sequences of Other Anti-sense Oligos**

Six oligos and their effects in a rabbit model were studied and the results of these studies are reported and discussed below. Five of these oligos were selected for this study to complement the data on Oligo I

(SEQ ID NO: 1) provided in Examples 1 to 4 above. This oligo is anti-sense to one region of the adenosine A_1 receptor mRNA. The oligos tested are identified as anti-sense Oligos I **(SEQ ID NO: 1)** and II **(SEQ ID NO: 7)** targeted to a different region of the adenosine A_1 receptor mRNA, Oligo V **(SEQ ID NO: 8)** targeted to the adenosine A_{2b} receptor mRNA, and anti-sense Oligos III and IV **(SEQ ID NOS: 9 and 10)** targeted to two different regions of the adenosine A_3 receptor mRNA. The sixth oligo (Oligo I-PD) is a phosphodiester version of Oligo I **(SEQ ID NO: 1)**. The design and synthesis of these anti-sense oligos was performed in accordance with Example 1 above.

(I) Anti-sense Oligo I

The anti-sense oligonucleotide I referred to in Examples 1 to 4 above is targeted to the human A_1 adenosine receptor mRNA (EPI 2010). Anti-sense oligo I is 21 nucleotide long, overlaps the initiation codon, and has the following sequence: 5'- GAT GGA GGG CGG CAT GGC GGG -3' **(:SEQ ID NO: 1)**. The oligo I was previously shown to abrogate the adenosine-induced bronchoconstriction in allergic rabbits, and to reduce allergen-induced airway obstruction and bronchial hyperresponsiveness (BHR), as discussed above and shown by Nyce, J. W. & Metzger, W. J., Nature, 385:721 (1977), the relevant portions of which reference are incorporated in their entireties herein by reference.

(II) Anti-sense Oligo II

A phosphorothioate anti-sense oligo **(SEQ ID NO: 7)** was designed in accordance with the invention to target the rabbit adenosine A_1 receptor mRNA region +936 to +956 relative to the initiation codon (start site). The anti-sense oligo II is 21 nucleotide long, and has the following sequence:5'-CTC GTC GCC GTC GCC GGC GGG-3' **(SEQ ID NO: 7)**.

(III) Anti-sense Oligo III

A phosphorothioate anti-sense oligo other than that provided in Example 1 above **(SEQ ID NO: 8)** was designed in accordance with the invention to target the anti-sense A_3 receptor mRNA region +3 to + 22 relative to the initiation codon start site. The anti-sense oligo III is 20 nucleotide long, and has the following sequence: 5'-GGG TGG TGC TAT TGT CGG GC-3' **(SEQ ID NO: 8)**.

(IV) Anti-sense Oligo IV

Yet another phosphorothioate anti-sense oligo **(SEQ ID NO: 9)** was designed in accordance with the invention to target the adenosine A_3 receptor mRNA region + 386 to + 401 relative to the initiation codon (start site). The anti-sense oligo IV is 15 nucleotide long, and has the following sequence: 5'-GGC CCA GGG CCA GCC-3' **(SEQ ID NO: 9)**.

(V) Anti-sense Oligo V

A phosphorothioate anti-sense oligo **(SEQ ID NO: 10)** was designed in accordance with the invention to target the adenosine A_{2b} receptor mRNA region -21 to -1 relative to the initiation codon (start site). The anti-sense oligonucleotide V is 21 nucleotide long, and has the following sequence: 5'-GGC CGG GCC AGC CGG GCC CGG-3' **(SEQ ID NO: 10)**.

(VI) A_1 Mismatch Oligos

Two different mismatched oligonucleotides having the following sequences were used as controls for anti-sense oligo I **(SEQ ID NO: 1)** described in Example 5 above: A_1 MM2 5'-GTA GGT GGC GGG CAA GGC GGG-3' **(SEQ ID NO: 12)**, and A_1 MM3 5'-GAT GGA GGC GGG CAT

GGC GGG-3' (**SEQ ID NO: 13**). Anti-sense oligo I and the two mismatch anti-sense oligos had identical base content and general sequence structure. Homology searches in GENBANK (release 85.0) and EMBL (release 40.0) indicated that the anti-sense oligo I was specific, not only for the human, but also for the rabbit, adenosine A_1 receptor genes, and that the mismatched controls were not candidates for hybridization with any known human or animal gene sequence.

(VII) Anti-sense Oligo A_1-PD (Oligo VI)

A phosphodiester anti-sense oligo (**Oligo VI; SEQ ID NO: 11**) having the same nucleotide sequence as Oligo I was designed as disclosed in the above-identified application. Anti-sense oligo I-PD is 21 nucleotide long, overlaps the initiation codon, and has the following sequence: 5'- GAT GGA GGG CGG CAT GGC GGG -3' (**SEQ ID NO: 11**).

(VIII) Controls

Each rabbit was administered 5.0 ml aerosolized sterile saline following the same schedule as for the anti-sense oligos in (II), (III), and (IV) above. The above are given as examples of G-protein coupled receptors. However, the method of reducing adenosine content is generally applicable to any gene and any target validation system in which the release of bioactive adenosine could obscure experimental data for actualizing adenosine receptors.

Example 7: Synthesis of Anti-sense Oligos

Phosphorothioate anti-sense oligos having the sequences described in (a) above, were synthesized on an Applied Biosystems Model 396 Oligonucleotide Synthesizer, and purified using NENSORB chromatography (DuPont, DE). TETD (tetraethylthiuram disulfide) was used as the sulfurizing agent during the synthesis. Anti-sense oligonucleotide II (**SEQ ID NO: 7**), anti-sense oligonucleotide III (**SEQ ID NO: 8**) and anti-sense oligonucleotide IV (**SEQ ID NO: 9**) were each synthesized and purified in this manner.

Example 8: Preparation of Allergic Rabbits

Neonatal New Zealand white Pasturella-free rabbits were immunized intraperitoneally within 24 hours of birth with 0.5 ml of 312 antigen units/ml house dust mite (D. *farinae*) extract (Berkeley Biologicals, Berkeley, CA) mixed with 10% kaolin as previously described (Metzger, W. J., in Late Phase Allergic Reactions, Dorsch, W., Ed., CRC Handbook, pp. 347-362, CRC Press, Boca Raton (1990); Ali, S., Metzger, W. J. and Mustafa, S. J., Am. J. Resp. Crit. Care Med. 149: 908 (1994)), the relevant portions of which are incorporated in their entireties here by reference. Immunizations were repeated weekly for the first month and then biweekly until the age of 4 months. These rabbits preferentially produce allergen-specific IgE antibody, typically respond to aeroallergen challenge with both an early and late-phase asthmatic response, and show bronchial hyper responsiveness (BHR). Monthly intraperitoneal administration of allergen (312 units dust mite allergen, as above) continues to stimulate and maintain allergen-specific IgE antibody and BHR. At 4 months of age, sensitized rabbits were prepared for aerosol administration as described by Ali et al. (Ali, S., Metzger, W. J. and Mustafa, S. J., Am. J. Resp. Crit. Care Med. 149 (1994)), the relevant section being incorporated in its entirety here by reference.

DOSE-RESPONSE STUDIES

Example 9: Experimental Setup

Aerosols of either adenosine (0-20 mg/ml), or anti-sense or one of two mismatch oligonucleotides (5 mg/ml) were separately prepared with an ultrasonic nebulizer (Model 646, DeVilbiss, Somerset, PA), which

produced aerosol droplets, 80% of which were smaller than 5:m in diameter. Equal volumes of the aerosols were administered directly to the lungs *via* an intratracheal tube. The animals were randomized, and administered aerosolized adenosine. Day 1 pre-treatment values for sensitivity to adenosine were calculated as the dose of adenosine causing a 50% loss of compliance (PC_{50} Adenosine). The animals were then administered either the aerosolized anti-sense or one of the mismatch anti-sense oligos via the intratracheal tube (5 mg/1.0 ml), for 2 minutes, twice daily for 2 days (total dose, 20 mg). Post-treatment PC_{50} values were recorded (post-treatment challenge) on the morning of the third day. The results of these studies are provided in Example 21 below.

Example 10: Cross-over Experiments

For some experiments utilizing anti-sense oligo I (**SEQ ID NO: 1**) and a corresponding mismatch control oligonucleotide A1MM2, following a 2 week interval, the animals were crossed over, with those previously administered the mismatch control A_1MM2, now receiving the anti-sense oligo I, and those previously treated with the anti-sense oligo I, now receiving the mismatch control A_1MM2 oligo. The number of animals per group was as follows. For mismatch A_1MM2 (Control 1), n=7, since one animal was lost in the second control arm of the experiment due to technical difficulties, for mismatch A_1MM3 n=4 (Control 2) and for A_1AS anti-sense oligo I, n=8. The A_1MM3 oligo-treated animals were analyzed separately and were not part of the cross-over experiment. The treatment methods and measurements employed following the cross-over were identical to those employed in the first arm of the experiment. In 6 of the 8 animals treated with the anti-sense oligo I (**SEQ ID NO: 1**), no PC_{50} value could be obtained for adenosine doses of up to 20 mg/ml, which is the limit of solubility of adenosine. Accordingly, the PC_{50} values for these animals were assumed to be 20 mg/ml for calculation purposes. The values given, therefore, represent a minimum figure for the effectiveness of the anti-sense oligonucleotides of the invention. Other groups of allergic rabbits (n=4 for each group) were administered 0.5 or 0.05 mg doses of the anti-sense oligo I (**SEQ ID NO: 1**), or the A_1MM2 oligo in the manner and according to the schedule described above (the total doses being 2.0 or 0.2 mg). The results of these studies are provided in Example 22 below.

Example 11: Anti-sense Oligo Formulation

Each one of anti-sense oligos were separately solubilized in an aqueous solution and administered as described for anti-sense oligo I (**SEQ ID NO: 1**) in (e) above, in four 5 mg aliquots (20 mg total dose) by means of a nebulizer via endotracheal tube, as described above. The results obtained for anti-sense oligo I and its mismatch controls confirmed that the mismatch controls are equivalent to saline, as described in Example 19 below and in Table 1 of Nyce & Metzger, Nature 385, 721-725 (1997), the contents of which are incorporated herein by reference. Because of this finding, saline was used as a control for pulmonary function studies employing anti-sense oligos II, III and IV (**SEQ ID NOS: 7, 8 and 9**).

Example 12: Specificity of Oligo I for Adenosine A_1 Receptor (Receptor Binding Studies)

Tissue from airway smooth muscle was dissected to primary, secondary and tertiary bronchi from rabbits which had been administered 20 mg oligo I (**SEQ ID NO: 1**) in 4 divided doses over a period of 48 hours as described above. A membrane fraction was prepared according to the method of Ali et al. (Ali, S., et al., Am. J. Resp. Crit. Care Med. 149: 908 (1994), the relevant section relating to the preparation of the membrane fraction is incorporated in its entirety hereby by reference). The protein content was determined by the method of Bradford and plasma membranes were incubated with 0.2 U/ml adenosine deaminase for 30 minutes at 37EC to remove endogenous adenosine. See, Bradford, M. M. Anal. Biochem. 72, 240-254 (1976), the relevant portion of which is hereby incorporated in its entirety by reference. The binding of

[^{3}H]DPCPX, [^{3}H]NPC17731, or [^{3}H]CGS-21680 was measured as described by Jarvis et al. See, Jarvis, M.F., et al., Pharmacol. Exptl. Ther. 251, 888-893 (1989), the relevant portion of which is fully incorporated herein by reference. Similar amounts of an oligo targeted to the bradykinin receptor 5'-GGTGATGTTGAGCATTTCGGC-3' (**SEQ ID NO: 14**) were administered to another group of animals. The results of this study are shown in Table 6 and discussed in Example 20 below.

Example 13: Pulmonary Function Measurements (Compliance c_{DYN} and Resistance)

At 4 months of age, the immunized animals were anesthetized and relaxed with 1.5 ml of a mixture of ketamine HCl (35 mg/kg) and acepromazine maleate (1.5 mg/kg) administered intramuscularly. After induction of anesthesia, allergic rabbits were comfortably positioned supine on a soft molded animal board. Salve was applied to the eyes to prevent drying, and they were closed. The animals were then intubated with a 4.0 mm intermediate high-low cuffed Murphy 1 endotracheal tube (Mallinckrodt, Glen Falls, NY), as previously described by Zavala and Rhodes. See, Zavala and Rhodes, Proc. Soc. Exp. Biol. Med. 144: 509-512 (1973), the relevant portion of which is incorporated herein by reference in its entirety. A polyethylene catheter of OD 2.4 mm (Becton Dickinson, Clay Adams, Parsippany NJ) with an attached thin-walled latex balloon was passed into the esophagus and maintained at the same distance (approximately 16 cm) from the mouth throughout the experiment. The endotracheal tube was attached to a heated Fleisch pneumotach (size 00; DEM Medical, Richmond, VA), and the flow (v) measured using a Validyne differential pressure transducer (Model DP-45-16-1927, Validyne Engineering, Northridge, CA), driven by a Gould carrier amplifier (Model 11-4113, Gould Electronics, Cleveland, OH). An esophageal balloon was attached to one side of the Validyne differential pressure transducer, and the other side was attached to the outflow of the endotracheal tube to obtain transpulmonary pressure (P_{tp}). The flow was integrated to yield a continuous tidal volume, and the measurements of total lung resistance (R_t) and dynamic compliance (C_{dyn}) were made at isovolumetric and zero flow points. The flow, volume and pressure were recorded on an eight channel Gould 2000 W high-frequency recorder and C_{dyn} was calculated using the total volume and the difference in P_{tp} at zero flow, and . R_t was calculated as the ratio of Ptp and V at midtidal lung volumes. These calculations were made automatically with the Buxco automated pulmonary mechanics respiratory analyzer (Model 6, Buxco Electronics, Sharon, CT), as previously described by Giles et al. See, Giles et al., Arch. Int. Pharmacodyn. Ther. 194: 213-232 (1971), the relevant portion of which describing these calculations is incorporated in toto hereby by reference. The results obtained upon administration of oligo II on allergic rabbits are shown and discussed in Example 26 below.

Example 14: Measurement of Bronchial Hyperresponsiveness (BHR)

Each allergic rabbit was administered histamine by aerosol to determine their baseline hyperresponsiveness. Aerosols of either saline or histamine were generated using a DeVilbiss nebulizer (DeVilbiss, Somerset, PA) for 30 seconds and then for 2 minutes at each dose employed. The ultrasonic nebulizer produced aerosol droplets of which 80% were <5 micron in diameter. The histamine aerosol was administered in increasing concentrations (0.156 to 80 mg/ml) and measurements of pulmonary function were made after each dose. The B4R was then determined by calculating the concentration of histamine (mg/ml) required to reduce the C_{dyn} 50% from baseline ($PC_{50\ Histamine}$).

Example 15: Cardiovascular Effect of Anti-sense Oligo I

The measurement of cardiac output and other cardiovascular parameters using CardiomaxJ utilizes the principal of thermal dilution in which the change in temperature of the blood exiting the heart after a venous injection of a known volume of cool saline is monitored. A single rapid injection of cool saline was

made into the right atrium via cannulation of the right jugular vein, and the corresponding changes in temperature of the mixed injectate and blood in the aortic arch were recorded via cannulation of the carotid artery by a temperature-sensing miniprobe. Twelve hours after the allergic rabbits had been treated with aerosols of oligo I (EPI 2010; **SEQ ID NO: 1**) as described in (d) above, the animals were anesthetized with 0.3 ml/kg of 80% Ketamine and 20% Xylazine. This time point coincides with previous data showing efficacy for **SEQ ID NO: 1**, as is clearly shown by Nyce & Metzger, (1997), supra, the pertinent disclosure being incorporated in its entirety here by reference. A thermocouple was then inserted into the left carotid artery of each rabbit, and was then advanced 6.5 cm and secured with a silk ligature. The right jugular vein was then cannulated and a length of polyethylene tubing was inserted and secured. A thermodilution curve was then established on a CardiomaxJ II (Columbus Instruments, Ohio) by injecting sterile saline at 20EC to determine the correctness of positioning of the thermocouple probe. After establishing the correctness of the position of the thermocouple, the femoral artery and vein were isolated. The femoral vein was used as a portal for drug injections, and the femoral artery for blood pressure and heart rate measurements. Once constant baseline cardiovascular parameters were established, CardiomaxJ measurements of blood pressure, heart rate, cardiac output, total peripheral resistance, and cardiac contractility were made.

Example 16: Duration of Action of Oligo I (SEQ ID NO: 1)

Eight allergic rabbits received initially increasing log doses of adenosine by means of a nebulizer via an intra-tracheal tube as described in (f) above, beginning with 0.156 mg/ml until compliance was reduced by 50% ($PC_{50\ Adenosine}$) to establish a baseline. Six of the rabbits then received four 5 mg aerosolized doses of (**SEQ ID NO: 1**) as described above. Two rabbits received equivalent amounts of saline vehicle as controls. Beginning 18 hours after the last treatment, the $PC_{50\ Adenosine}$ values were tested again. After this point, the measurements were continued for all animals each day, for up to 10 days. The results of this study are discussed in Example 25 below.

Example 17: Reduction of Adenosine A_{2b} Receptor Number by Anti-sense Oligo V

Sprague Dawley rats were administered 2.0 mg respirable anti-sense oligo V (**SEQ ID NO: 10**) three times over two days using an inhalation chamber as described above. Twelve hours after the last administration, lung parenchymal tissue was dissected and assayed for adenosine A_{2b} receptor binding using [311]-NECA as described by Nyce & Metzger (1997), supra. Controls were conducted by administration of equal volumes of saline. The results are significant at p<0.05 using Student's paired t test, and are discussed in Example 28 below.

Example 18: Comparison of Oligo I & Corresponding Phosphodiester Oligo VI (SEQ ID NO: 11)

Oligo I (**SEQ ID NO: 1**) countered the effects of adenosine and eliminated sensitivity to it for adenosine amounts up to 20 mg adenosine/5.0 ml (the limit of solubility of adenosine). Oligo VI (**SEQ ID NO: 11**), the phosphodiester version of the oligonucleotide sequence, was completely ineffective when tested in the same manner. Both compounds have identical sequence, differing only in the presence of phosphorothioate residues in Oligo I (**SEQ ID NO: 1**), and were delivered as an aerosol as described above and in Nyce & Metzger (1997), supra. Significantly different at p<0.001, Student's paired t test. The results are discussed in Example 29 below.

RESULTS OBTAINED FOR ANTI-SENSE OLIGO I - (SEQ ID NO: 1)

Example 19: Results of Prior Work

The nucleotide sequence and other data for anti-sense oligo I (**SEQ ID NO: 1**), which is specific for the adenosine A_1 receptor, were provided above. The experimental data showing the effectiveness of oligo I in down regulating the receptor number and activity were also provided above. Further information on the characteristics and activities of anti-sense oligo I is provided in Nyce, J. W. and Metzger, W. J., Nature 385:721 (1997), the relevant parts of which relating to the following results are incorporated in their entireties herein by reference. The Nyce & Metzger (1997) publication provided data showing that the anti-sense oligo I (**SEQ ID NO: 1**):

(1) The anti-sense oligo I reduces the number of adenosine A_1 receptors in the bronchial smooth muscle of allergic rabbits in a dose-dependent manner as may be seen in Table 6 below.

(2) Anti-sense Oligo I attenuates adenosine-induced bronchoconstriction and allergen-induced bronchoconstriction.

(3) The Oligo I attenuates bronchial hyperresponsiveness as measured by PC_{50} histamine, a standard measurement to assess bronchial hyperresponsiveness. This result clearly demonstrates anti-inflammatory activity of the anti-sense oligo I as is shown in Tables 4, 5 and 6.

(4) As expected, because it was designed to target it, the anti-sense oligo I is totally specific for the adenosine A_1 receptor, and has no effect at all at any dose on either the very closely related adenosine A_2 receptor or the related bradykinin B_2 receptor. This is seen in Table 6 below.

(5) In contradistinction to the above effects of the Oligo I, the mismatch control molecules MM2 and MM3 (**SEQ ID NO: 12** and **SEQ ID NO: 13**) which have identical base composition and molecular weight but differed from the anti-sense oligo I (**SEQ ID NO: 1**) by 6 and 2 mismatches, respectively. These mismatches, which are the minimum possible while still retaining identical base composition, produced absolutely no effect upon any of the targeted receptors (A_1, A_2 or B_2).

These results, along with a complete lack of prior art on the use of anti-sense oligonucleotides, such as oligo I, targeted to the adenosine A_1 receptor, are unexpected results. The showings presented in this patent clearly enable and demonstrate the effectiveness, for its intended use, of the validation method employing agents and targeted to genes or mRNA associated with a function or end point associated with pulmonary functions, such as airway blockage, bronchoconstriction, pulmonary inflammation and allergy(ies), and the like.

Example 20: Oligo I Significantly Reduces Response to Adenosine Challenge

The receptor binding experiment is described in Example 12 above, and the results shown in Table 6 below which shows the binding characteristics of the adenosine A_1-selective ligand [$_3$H]DPCPX and the bradykinin B_2-selective ligand [^{3}H]NPC 17731 in membranes isolated from airway smooth muscle of A_1 adenosine receptor and B_2 bradykinin receptor anti-sense- and mismatch-treated allergic rabbits.

Table 6: Binding Characteristics of Three Anti-Sense Oligos

Treatment[1]	A_1 receptor		B_2 receptor	
	Kd	B_{max}	Kd	B_{max}
Adenosine A_1	**Receptor**			
20 mg	0.36±0.029 nM	19±1.52 fmoles*	0.39±0.031 nM	14.8±0.99fmoles
2 mg	0.38±0.030 nM	32±2.56 fmoles*	0.41±0.028 nM	15.5±1.08 fmoles
0.2 mg	0.37±0.030 nM	49±3.43 fmoles	0.34±0.024 nM	15.0±1.06 fmoles
A_1MM1	**(Control)**			
20 mg	0.34±0.027 nM	52.0±3.64 fmoles	0.35±0.024 nM	14.0±1.0 fmoles
2 mg	0.37±0.033 nM	51.8±3.88 fmoles	0.38±0.028 nM	14.6±1.02 fmoles
B_2A (Bradykinin	**Receptor)**			
20 mg	0.36±0.028 nM	45.0±3.15 fmoles	0.38±0.027 nM	8.7±0.62 fmoles*
2 mg	0.39±0.035 nM	44.3±2.90 fmoles	0.34±0.024 nM	11.9±0.76 fmoles**
0.2 mg	0.40±0.028 nM	47.0±3.76 fmoles	0.35±0.028 nM	15.1±1.05 fmoles
B_2MM (Control)				
20 mg	0.39±0.031 nM	42.0±2.94 fmoles	0.41±0.029 nM	14.0±0.98 fmoles
2 mg	0.41±0.035 nM	40.0±3.20 fmoles	0.37±0.030 nM	14.8±0.99 fmoles
0.2 mg	0.37±0.029 nM	43.0±3.14 fmoles	0.36±0.025 nM	15.1±1.35 fmoles
Saline Control	0.37±0.041	46.0±5.21	0.39±0.047 nM	14.2±1.35 fmoles

[1] Refers to total oligo administered in four equivalently divided doses over a 48 hour period. Treatments and analyses were performed as described in methods. Significance was determined by repeated-measures analysis of variance (ANOVA), and Tukey's protected t test. n = 4-6 for all groups.
* Significantly different from mismatch control- and saline-treated groups, $p<0.001$;
**Significantly different from mismatch control- and saline-treated groups, $p<0.05$.

Example 21: Dose-response Effect of Oligo I

Anti-sense oligo I (**SEQ ID NO: 1**) was found to reduce the effect of adenosine administration to the animal in a dose-dependent manner over the dose range tested as shown in Table 7 below.

Table 7: Dose-Response Effect to Anti-sense Oligo I

Total Dose (mg)	PC_{50} Adenosine (mg Adenosine)
Anti-sense Oligo I	
0.2	8.32"7.2
2.0	14.0"7.2
20	19.5"0.34
A_1MM2 oligo (control)	
0.2	2.51±0.46
2.0	3.13± 0.71
20	3.25± 0.34

The above results were studied with the Student's paired t test and found to be statistically different, p=0.05

The oligo I (**SEQ ID NO: 1**), an anti-adenosine A_1 receptor oligo, acts specifically on the adenosine A_1 receptor, but not on the adenosine A_2 receptors. These results stem from the treatment of rabbits with anti-sense oligo I (**SEQ ID NO: 1**) or mismatch control oligo (**SEQ ID NO: 12**; A_1MM2) as described in Example 9 above and in Nyce & Metzger (1997), supra (four doses of 5 mg spaced 8 to 12 hours apart via nebulizer via endotracheal tube), bronchial smooth muscle tissue excised and the number of adenosine A_1 and adenosine A_2 receptors determined as reported in Nyce & Metzger (1997), supra.

Example 22: Specificity of Oligo I (SEQ ID NO: 1) for Target Gene Product

Oligo I (**SEQ ID NO: 1**) is specific for the adenosine A_1 receptor whereas its mismatch controls had no activity. Figure 1 depicts the results obtained from the cross-over experiment described in Example 10 above and in Nyce & Metzger (1997), supra. The two mismatch controls (**SEQ ID NO: 12** and **SEQ ID NO: 13**) evidenced no effect on the $PC_{50\ Adenosine}$ value. On the contrary, the administration of anti-sense oligo I (**SEQ ID NO: 1**) showed a seven-fold increase in the $PC_{50\ Adenosine}$ value. The results clearly indicate that the anti-sense oligo I (**SEQ ID NO: 1**) reduces the response (attenuates the sensitivity) to exogenously administered adenosine when compared with a saline control. The results provided in Table 6 above clearly establish that the effect of the anti-sense oligo I is dose dependent (see, column 3 of Table 6). The Oligo I was also shown to be totally specific for the adenosine A_1 receptor, (see, top 3 rows of Table 6), inducing no activity at either the closely related adenosine A_2 receptor or the bradykinin B_2 receptor (see, lines 8-10 of Table 6 above). In addition, the results shown in Table 6 establish that the anti-sense oligo I (**SEQ ID NO: 1**) decreases sensitivity to adenosine in a dose dependent manner, and that it does this in an anti-sense oligo-dependent manner since neither of two mismatch control oligonucleotides (A_1MM2: **SEQ ID NO: 12** and A_1MM3: **SEQ ID NO: 13**) show any effect on $PC_{50\ Adenosine}$ values or on attenuating the number of adenosine A_1 receptors.

Example 23: Effect on Aeroallergen-induced Bronchoconstriction & Inflammation

The Oligo I (**SEQ ID NO: 1**) was shown to significantly reduce the histamine-induced effect in the rabbit model when compared to the mismatch oligos. The effect of the anti-sense Oligo I (SEQ ID No:1) and the mismatch oligos (A_1MM2, **SEQ ID NO: 12** and A_1MM3, **SEQ ID NO: 12**) on allergen-induced airway obstruction and bronchial hyperresponsiveness was assessed in allergic rabbits. The effect of the anti-sense oligo I (**SEQ ID NO: 1**) on allergen-induced airway obstruction was assessed. As calculated from the area under the plotted curve, the anti-sense oligo I significantly inhibited allergen-induced airway obstruction when compared with the mismatched control (55%, $p<0.05$; repeated measures ANOVA, and Tukey's t test). A complete lack of effect was induced by the mismatch oligo A_1MM2 (Control) on allergen induced airway obstruction. The effect of the anti-sense oligo I (**SEQ ID NO: 1**) on allergen-induced BHR was determined as above. As calculated from the $PC_{50\ Histamine}$ value , the anti-sense oligo I (**SEQ ID NO: 1**) significantly inhibited allergen-induced BHR in allergic rabbits when compared to the mismatched control (61%, $p<0.05$; repeated measures ANOVA, Tukey's t test). A complete lack of effect of the A_1MM mismatch control on allergen-induced BHR was observed. The results indicated that anti-sense oligo I (**SEQ ID NO: 1**) is effective to protect against aeroallergen-induced bronchoconstriction (house dust mite). In addition, the anti-sense oligo I (**SEQ ID NO: 1**) was also found to be a potent inhibitor of dust mite-induced bronchial hyper responsiveness, as shown by its effects upon histamine sensitivity which indicates anti- inflammatory activity for anti-sense oligo I (**SEQ ID NO: 1**).

Example 24: Low A Content Anti-sense Oligo I is Free of Deleterious Side Effects

The Oligo I (**SEQ ID NO: 1**) was shown to be free of side effects that might be toxic to the recipient. No changes in arterial blood pressure, cardiac output, stroke volume, heart rate, total peripheral resistance or heart contractility (dPdT) were observed following administration of 2.0 or 20 mg oligo I (**SEQ ID NO: 1**). The addition, the results of the measurement of cardiac output (CO), stroke volume (SV), mean arterial pressure (MAP), heart rate (HR), total peripheral resistance (TPR), and contractility (dPdT) with a CardiomaxJ apparatus (Columbus Instruments, Ohio) were assessed. These results evidenced that oligo I (**SEQ ID NO: 1**) has no detrimental effect upon critical cardiovascular parameters. More particularly, this oligo does not cause hypotension. This finding is of particular importance because other phosphorothioate anti-sense oligonucleotides have been shown in the past to induce hypotension in some model systems. Furthermore, the adenosine A_1 receptor plays an important role in sinoatrial conduction within the heart. Attenuation of the adenosine A_1 receptor by anti-sense oligo I (**SEQ ID NO: 1**) might be expected to result, therefore, in deleterious extrapulmonary activity in response to the down regulation of the receptor. This is not the case. The anti-sense oligo I (**SEQ ID NO: 1**) does not produce any deleterious intrapulmonary effects and renders the administration of the low doses of the present anti-sense oligo free of unexpected, undesirable side effects. This demonstrates that when oligo I (**SEQ ID NO: 1**) is administered directly to the lung, it does not reach the heart in significant quantities to cause deleterious effects. This is in contrast to traditional adenosine receptor antagonists like theophylline which do escape the lung and can cause deleterious, even life-threatening effects outside the lung.

Example 25: Long Lasting Effect of Oligo I

The Oligo I (**SEQ ID NO: 1**) evidenced a long lasting effect as evidenced by the PC_{50} and Resistance values obtained upon its administration prior to adenosine challenge. The duration of the effect was measured for with respect to the PC_{50} of adenosine anti-sense oligo I when administered in four equal doses of 5 mg each by means of a nebulizer via an endotracheal tube, as described above. The effect of the agent is significant over days 1 to 8 after administration. When the effect of the anti-sense oligo I (**SEQ ID NO: 1**) had disappeared, the animals were administered saline aerosols (controls), and the $PC_{50\ Adenosine}$ values for all animals were measured again. Saline-treated animals showed base line PC_{50} adenosine values (n=6). The duration of the effect (with respect to Resistance) was measured for six allergic rabbits which were administered 20 mg of anti-sense oligo I (**SEQ ID NO: 1**) as described above, upon airway resistance measured as also described above. The mean calculated duration of effect was 8.3 days for both PC_{50} adenosine ($p<0.05$) and resistance ($p<0.05$). These results show that anti-sense oligo I (**SEQ ID NO: 1**) has an extremely long duration of action, which is completely unexpected.

Example 26: Adenosine-free Anti-sense Oligo II Better than Anti-sense Oligo I

Anti-sense oligo II, targeted to a different region of the adenosine A_1 receptor mRNA, was found to be highly active against the adenosine A_1-mediated effects. The experiment measured the effect of the administration of anti-sense oligo II (**SEQ ID NO: 7**) upon compliance and resistance values when 20 mg anti-sense oligo II or saline (control) were administered to two groups of allergic rabbits as described above. Compliance and resistance values were measured following an administration of adenosine or saline as described above in Example 13. The effect of the anti-sense oligo of the invention was different from the control in a statistically significant manner, $p<0.05$ using paired t-test, compliance; $p<0.01$ for resistance.

The results showed that anti-sense oligo II (**SEQ ID NO: 7**), which targets the adenosine A_1 receptor and which contains no adenosine, effectively maintains compliance and reduces resistance upon adenosine challenge. In fact, the adenosine-free anti-sense Oligo II is more potent than the low adenosine anti-sense Oligo I (**SEQ ID NO: 1**). Because it contains no adenosine, it will not liberate adenosine during degradation, and hence it will not contribute to activating adenosine receptors.

Example 27: Anti-sense Oligos III and IV

Oligos III (**SEQ ID NO: 8**) and IV (**SEQ ID NO: 9**) were shown to be in fact specifically targeted to the adenosine A_3 receptor by their effect on reducing inflammation and the number of inflammatory cells present upon separate administration of 20 mg of the anti-sense oligos III (**SEQ ID NO: 8**) and IV (**SEQ ID NO: 9**) to allergic rabbits as described above. The number of inflammatory cells was determined in their bronchial lavage fluid 3 hours later by counting at least 100 viable cells per lavage. The effect of anti-sense oligos III (**SEQ ID NO: 8**) and IV (**SEQ ID NO: 9**) upon granulocytes, and upon total cells in bronchial lavage were assessed following exposure to dust mite allergen. The results showed that the anti-sense oligo IV (**SEQ ID NO: 9**) and anti-sense oligo III (**SEQ ID NO: 8**) are very potent anti-inflammatory agents in the asthmatic lung following exposure to dust mite allergen. As is known in the art, granulocytes, especially eosinophils, are the primary inflammatory cells of asthma, and the administration of anti-sense oligos III (**SEQ ID NO: 8**) and IV (**SEQ ID NO: 9**) reduced their numbers by 40% and 66%, respectively. Furthermore, anti-sense oligos IV (**SEQ ID NO: 9**) and III (**SEQ ID NO: 8**) also reduced the total number of cells in the bronchial lavage fluid by 40% and 80%, respectively. This is also an important indicator of anti-inflammatory activity by the present anti-adenosine A_3 agents of the invention. Inflammation is known to underlie bronchial hyperresponsiveness and allergen-induced bronchoconstriction in asthma. Both anti-sense oligonucleotides III (**SEQ ID NO: 8**) and IV (**SEQ ID NO: 9**), which are targeted to the adenosine A_3 receptor, are representative of an important new class of anti-inflammatory agents which may be designed to specifically target the lung receptors of each species.

Example 28: Anti-sense Oligo V

The anti-sense oligo V (**SEQ ID NO: 10**) , targeted to the adenosine A_{2b} adenosine receptor mRNA was shown to be highly effective at countering adenosine A_{2b}-mediated effects and at reducing the number of adenosine A_{2b} receptors present to less than half.

Example 29: Unexpected Superiority of Substituted over Phosphodiester-residue Oligo I-DS (SEQ ID NO: 11)

Oligos I (**SEQ ID NO: 1**) and I-DS (**SEQ ID NO: 11**) were separately administered to allergic rabbits as described above, and the rabbits were then challenged with adenosine. The phosphodiester oligo I-DS (**SEQ ID NO: 11**) was statistically significantly less effective in countering the effect of adenosine whereas oligo I (**SEQ ID NO: 1**) showed high effectiveness, evidencing a $PC_{50\ Adenosine}$ of 20 mg.

Example 30: Adenosine Containing Mononucleotides have Adenosine Receptor Activity

This example demonstrates that in vivo break down products of anti-sense oligonucleotides such as ribonucleoside monophosphates, e.g. dAMP act at adenosine receptors. When adenosine and adenosine monophosphate (dAMP) were separately administered to experimental animals at different doses of up to 10 mg/ml, both compounds have a similar effect in reducing % compliance as shown in Figure 1. The effect in

both cases increases with the dose whereas a saline control shows no effect. These results show that adenosine nucleosides as well as adenosine itself, interact with adenosine receptors.

<u>Example 31</u>: Breakdown of Adenosine Containing Nucleic Acid Produce Adenosine Receptor Activity

As a further test, randomer phosphorothioate anti-sense oligonucleosides were administered to rabbits to determine if they were degraded in vivo and released adenosine nucleosides capable of interacting with adenosine receptors. Asthmatic rabbits were separately administered saline (control), an adenosine containing randomer (?) and a desAdenosine randomer (C). The randomers used were a desAdenosine randomer consisting of random sequences of guanine, cytosine and thymidine and an adenosine containing randomer consisting of guanine, cytosine and adenosine. The results shown in Figure 2 clearly indicate that adenosine containing oligonucleotides release adenosine and/or adenosine nucleosides upon degradation, and that the adenosine compounds interact with adenosine receptors, while desAdenosine oligonucleotides do not. The release of adenosine nucleosides as degradation products of anti-sense oligonucleotides, thus, would confuse experimental results when assessing the effects of anti-sense knock out experiments for Target Validation. This experiment shows the necessity of using desAdenosine anti-sense (no A or low A) oligonucleotides in Target Validation studies.

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

WHAT IS CLAIMED AS NOVEL AND UNOBVIOUS
IN UNITED STATES LETTERS PATENT IS:

1. A method of determining the existence of a correlation between a function of a disease or condition and a gene or mRNA encoding a target polypeptide suspected of being associated with a disease or condition, comprising

obtaining oligonucleotides (oligos) consisting of up to about 15% adenosine (A), and which is anti-sense to a target selected from the group consisting of target genes and their corresponding mRNAs, genomic and mRNA flanking regions selected from the group consisting of 3' and 5' intron-exon borders and the juxta-section between coding and non-coding regions, and all mRNA segments encoding polypeptides associated with a pre-selected disease or condition;

selecting amongst the oligos one that significantly inhibits or ablates expression of the polypeptide encoded by the mRNA upon in vitro hybridization to the target mRNA;

administering to a subject an amount of the selected oligo effective for in vivo hybridization to the target mRNA; and

assessing a subject's function that is associated with the disease or condition before and after administration of the oligo; wherein a change in the function's value greater than about 70% indicates a positive correlation, between about 40 and about 70% a possible correlation, and below about 30% a lack of correlation.

2. The method of claim 1, wherein the anti-sense oligos are constructed by selecting target fragments

having at least 4 contiguous nucleic acids selected from the group consisting of G and C and obtaining a first oligonucleotide 4 to 60 nucleotides long which comprises the selected fragment and has a C and G content of up to about 15%, and/or

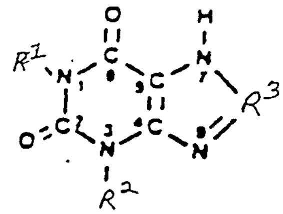
having a desirable type and/or extent of activity.

3. The method of claim 1, further comprising, when the anti-sense fragment comprises at least one A, substituting at least one A with an alternative base (B) selected from the group consisting of heteroaromatic bases which bind to thymidine (T) but have less than about 0.3 of A's adenosine A_1, A_{2a}, A_{2b} and A_3 receptor agonist or antagonist activity.

4. The method of claim 3, wherein the heteroaromatic bases are selected from the group consisting of pyrimidines and purines, which may be substituted by O, halo, NH_2, SH, SO, SO_2, SO_3, COOH and branched and fused primary and secondary amino, alkyl, alkenyl, alkynyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, alkoxy, alkenoxy, acyl, cycloacyl, arylacyl, alkynoxy, cycloalkoxy, aroyl, arylthio, arylsulfoxyl, halocycloalkyl, alkylcycloalkyl, alkenylcycloalkyl, alkynylcycloalkyl, haloaryl, alkylaryl, alkenylaryl, alkynylaryl, arylalkyl, arylalkenyl, arylalkynyl, arylcycloalkyl, which may be further substituted by O, halo, NH_2, primary, secondary and tertiary amine, SH, SO, SO_2, SO_3, cycloalkyl, heterocycloalkyl and heteroaryl.

5. The method of claim 4, wherein the pyrimidines and purines are substituted at positions 1, 2, 3, 4, 7 and 8.

6. The method of claim 4, wherein the pyrimidines and purines are selected from the group consisting of theophylline, caffeine, dyphylline, etophylline, acephylline piperazine, bamifylline, enprofylline and xanthine having the chemical formula



wherein R^1 and R^2 are independently H, alkyl, alkenyl or alkynyl and R^3 is H, aryl, dicycloalkyl, dicycloalkenyl, dicycloalkynyl, cycloalkyl, cycloalkenyl, cycloalkynyl, O-cycloalkyl, O-cycloalkenyl, O-cycloalkynyl, NH_2-alkylamino-ketoxyalkyloxy-aryl and mono and dialkylaminoalkyl-N-alkylamino-SO_2 aryl.

7. The method of claim 1, wherein the anti-sense oligo has an adenosine content of about 0 to about 12%.

8. The method of claim 7, wherein the oligo consists of up to about 5% A.

9. The method of claim 8, wherein the oligo is A-free.

10. The method of claim 1, wherein one A is substituted by an alternative base selected from the group consisting of heteroaromatic bases which bind to a thymidine base but have antagonist or agonist activity of less than about 0.5 of the adenosine base agonist or antagonist activity at the adenosine A_1, A_{2a}, A_{2b} and A_3 receptors.

11. The method of claim 10, wherein all As are substituted by an alternative base selected from the group consisting of heteroaromatic bases which bind to a thymidine base but have activity less than about 0.3 of the adenosine base agonist or antagonist activity at the adenosine A_1, A_{2a} A_{2b} and A_3 receptors.

12. The method of claim 6, wherein the universal base is selected from the group consisting of 3-nitropyrrole-2'-deoxynucleoside, 5-nitro-indole, 2-deoxyribosyl-(5-nitroindole), 2-deoxyribofuranosyl-(5-nitroindole), 2'-deoxyinosine, 2'-deoxynebularine, 6H, 8H-3,4-dihydropyrimido [4,5-c] oxazine-7-one or 2-amino-6-methoxyaminopurine.

13. The method of claim 1, where a methylated cytosine (^mC) is substituted instead of C in at least one CpG dinucleotide if present in the oligo(s).

14. The method of claim 1, wherein at least one nucleotide residue of the anti-sense oligonucleotide(s) is a residue selected from the group consisting of methylphosphonate, phosphotriester, phosphorothioate, phosphorodithioate, boranophosphate, formacetal, thioformacetal, thioether, carbonate, carbamate, sulfate, sulfonate, sulfamate, sulfonamide, sulfone, sulfite, sulfoxide, sulfide, hydroxylamine, methylene(methyimino), (MMI), methoxymethyl (MOM), methoxyethyl (MOE), methyleneoxy (methylimino) (MOMA), methoxy methyl (MOM), 2'-O-methyl, phosphoramidate, and C-5 substituted residues, and combinations thereof.

15. The agent of claim 14, wherein all nucleotide linking residues are substituted.

16. The method of claim 1, wherein the anti-sense oligo comprises about 7 to 60 mononucleotides.

17. The method of claim 1, wherein the anti-sense oligo is linked to an agent selected from the group consisting of cell internalized or up-taken agent(s) and cell targeting agents.

18. The method of claim 17, wherein the cell internalized or up taken agent is selected from the group consisting of transferrin, asialoglycoprotein and streptavidin.

19. The method of claim 18, wherein the nucleic acid is linked to a vector.

20. The method of claim 19, wherein the vector comprises a prokaryotic or eukaryotic vector.

21. The method of claim 1, wherein the anti-sense oligo is administered to the lung, brain, heart, kidney, tumor, blood, skin, eye, scalp, nose panages, testes, cervix, oral cavity, pharynx, esophagus, small or large intestine, synovial tissue, muscle tissue, ovaries, ear canal or in vitro.

22. The method of claim 1, wherein the disease or condition is a disease or condition afflicting the lung, brain, heart, kidney, tumor, blood, immune system, skin, eye, scalp, nose panages, testes, cervix, oral cavity, pharynx, esophagus, small or large intestine, synovial tissue, muscle tissue, ovaries, and ear canal.

23. The method of claim 22, wherein the disease or condition is a disease or condition afflicting the lung.

24. The method of claim 22, wherein the disease or condition is associated with bronchoconstriction, lung inflammation and/or allergy(ies).

25. The method of claim 22, wherein the disease or condition is a disease or condition afflicting the brain, or associated with brain activity.

26. The method of claim 22, wherein the disease or condition is associated with immune dysfunction.

27. The method of claim 26, wherein the target is selected from the group consisting of immunoglobulins, antibody receptors, cytokines, cytokine receptors, gene(s) and the corresponding mRNA(s) encoding them, the genes and mRNA flanking regions and intron and exon borders.

28. The method of claim 22, wherein the disease or condition is a disease or condition afflicting the cardiovascular system.

29. The method of claim 22, wherein the disease or condition is a disease or condition associated with the gastrointestinal system.

30. The method of claim 22, wherein the disease or condition is associated with a malignancy or cancer.

31. The method of claim 30, wherein the target is selected from the group consisting of immunoglobulins and antibody receptors, gene(s) and mRNA(s) encoding them, genes and mRNAs associated with oncogenes, and genomic and mRNA flanking regions and exon and intron borders.

32. The method of claim 1, wherein the composition is administered in vitro, orally, intracavitarily, intranasally, intraanally, intravaginally, intrauterally, intrachranially, pulmonarily, intrarenally, intranodularly, intraarticularly, intraotically, intralymphatically, transdermally, intrabucally, intravenously, subcutaneously, intramuscularly, intratumorously, intraglandularly, intraocularly, intracranial, into an organ, intravascularly, intrathecally, by implantation, by inhalation, intradermally, intrapulmonarily, into the ear, into the heart, by slow release, by sustained release and by a pump.

33. The method of claim 1, wherein the target is selected from the group consisting of genes and mRNAs encoding polypeptides selected from the group consisting of transcription factors, stimulating and activating factors, cytokines and their receptors, interleukins, interleukin receptors, chemokines, chemokine receptors, endogenously produced specific and non-specific enzymes, immunoglobulins, antibody receptors, central nervous system (CNS) and peripheral nervous and non-nervous system receptors, CNS and peripheral nervous and non-nervous system peptide transmitters, adhesion molecules, defensines, growth factors, vasoactive peptides, peptide receptors and binding protein, and genes and mRNAs corresponding to oncogenes.

34. The method of claim 1, wherein the anti-sense oligo(s) are produced by

selecting a target from the group consisting of polypeptides associated with a disease(s) and/or condition(s) afflicting lung airways, genes and RNAs encoding them, the genomic and mRNA flanking regions and the gene(s) and mRNA(s) intron and exon borders;

obtaining the sequence of a mRNA(s) selected from the group consisting of mRNAs corresponding to the target gene(s) and mRNAs encoding the target polypeptide(s), genomic and mRNA flanking regions and the genes and mRNAs intron and exon borders;

selecting at least one segment of the mRNA(s);

synthesizing one or more oligo anti-sense to the selected mRNA segment(s); and

substituting, if necessary, an alternative base(s) for one or more A(s) to reduce the content of A present in the oligo to up to about 15% of all nucleotides.

35. The method of claim 1, wherein the target gene is selected from the group consisting of target genes and mRNAs encoding polypeptides selected from the group consisting of transcription factors, stimulating and activating factors, interleukins, interleukin receptors, chemokines, chemokine receptors, endogenously produced specific and non-specific enzymes, immunoglobulins, antibody receptors, central nervous system (CNS) and peripheral nervous and non-nervous system receptors, CNS and peripheral nervous and non-nervous system peptide transmitters and their receptors, adhesion molecules, defensines, growth factors, vasoactive peptides and their receptors, and binding proteins, and target genes and mRNAs corresponding to oncogenes, and their flanking regions and intron and exon borders.

36. The method of claim 35, wherein the encoded polypeptides are selected from the group consisting of NfκB Transcription Factor, Interleukin-8 Receptor (IL-8 R), Interleukin 5 Receptor (IL-5 R), Interleukin 4 Receptor (IL-4 R), Interleukin 3 Receptor (IL-3 R), Interleukin-1β (IL-1β), Interleukin 1β Receptor (IL- 1β R), Eotaxin, Tryptase, Major Basic Protein, β2-adrenergic Receptor Kinase, Endothelin Receptor A, Endothelin Receptor B, Preproendothelin, Bradykinin B2 Receptor, IgE High Affinity Receptor, Interleukin 1 (IL-1), Interleukin 1 Receptor (IL-1 R), Interleukin 9 (IL-9), Interleukin-9 Receptor (IL-9 R), Interleukin 11 (IL-11), Interleukin-11 Receptor (IL-11 R), Inducible Nitric Oxide Synthase, Cyclooxygenase (COX), Intracellular Adhesion Molecule 1 (ICAM-1) Vascular Cellular Adhesion Molecule (VCAM), Rantes, Endothelial Leukocyte Adhesion Molecule (ELAM-1), Monocyte Activating Factor, Neutrophil Chemotactic Factor, Neutrophil Elastase, Defensin 1, 2 and 3, Muscarinic Acetylcholine Receptors, Platelet Activating Factor, Tumor Necrosis Factor α, 5-lipoxygenase, Phosphodiesterase IV, Substance P, Substance P Receptor, Histamine Receptor, Chymase, CCR-1 CC Chemokine Receptor, CCR-2 CC Chemokine Receptor, CCR-3 CC Chemokine Receptor, CCR-4 CC Chemokine Receptor, CCR-5 CC Chemokine Receptor, Prostanoid Receptors, GATA-3 Transcription Factor, Neutrophil Adherence Receptor, MAP Kinase, Interleukin-9 (IL-9), NFAT Transcription Factors, STAT 4, MIP-1α, MCP-2, MCP-3, MCP-4, Cyclophillins, Phospholipase A2, Basic Fibroblast Growth Factor, Metalloproteinase, CSBP/p38 MAP Kinase, Tryptose Receptor, PDG2, Interleukin-3 (IL-3), Interleukin-1β (IL-1β), Cyclosporin A-Binding Protein, FK5-Binding Protein, α4β1 Selectin, Fibronectin, α4β7 Selectin, Mad CAM-1, LFA-1 (CD11a/CD18), PECAM-1, LFA-1 Selectin, C3bi, PSGL-1, E-Selectin, P-Selectin, CD-34, L-Selectin, p150,95, Mac-1 (CD11b/CD18), Fucosyl transferase, VLA-4, CD-18/CD11a, CD11b/CD18, ICAM2 and ICAM3, C5a, CCR3 (Eotaxin Receptor), CCR1, CCR2, CCR4, CCR5, LTB-4, AP-1 Transcription Factor, Protein kinase C, Cysteinyl Leukotriene Receptor, Tachychinnen Receptors (tach R), IκB Kinase 1 & 2, STAT 6, c-mas and NF-Interleukin-6 (NF-IL-6), and their flanking regions and intron and exon borders.

37. The method of claim 1, wherein the target gene encodes a G-protein or a G-protein coupled receptor.

38. The method of claim 1, wherein the target gene encodes a calcium channel protein or receptor, a sodium channel protein or receptor, a potassium channel protein or receptor, or a chloride channel protein or receptor.

39. The method of claim 1, wherein the target gene encodes a neurotransmitter receptor or a neurohormone receptor.

40. The method of claim 1, wherein the target gene encodes a neuropeptide or neuropeptide

receptor.

41. The method of claim 1, further comprising repeating all steps with the separate administration of further oligo(s) which are anti-sense to further targets whose functions are suspected of being associated with the first target; repeating the administration and assessment steps with the joint administration oligos targeted to the first and further targets; and comparing the results obtained with those obtained separately for each target; wherein when the combined oligo effects are about 20% or more greater than that with each oligo, it may be said there is a positive association between the first and further oligo(s), when the results are within about 20% of that of one oligo it may be said that there is no association, and when the results are less than about 20% lower than with an individual oligo it may be said that there is a negative association between them.

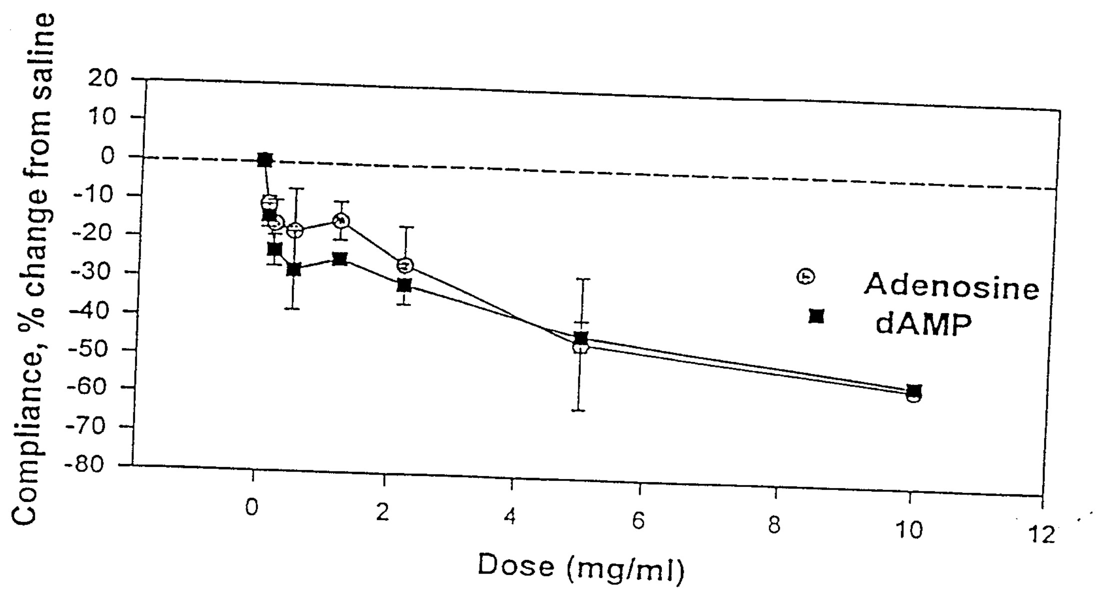


Figure 1

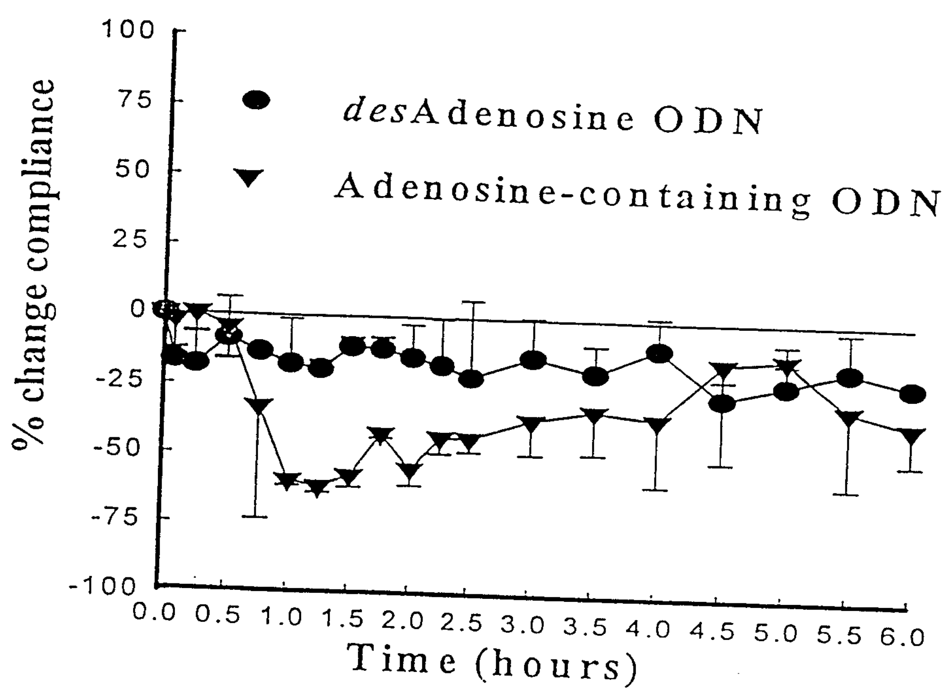


Figure 2

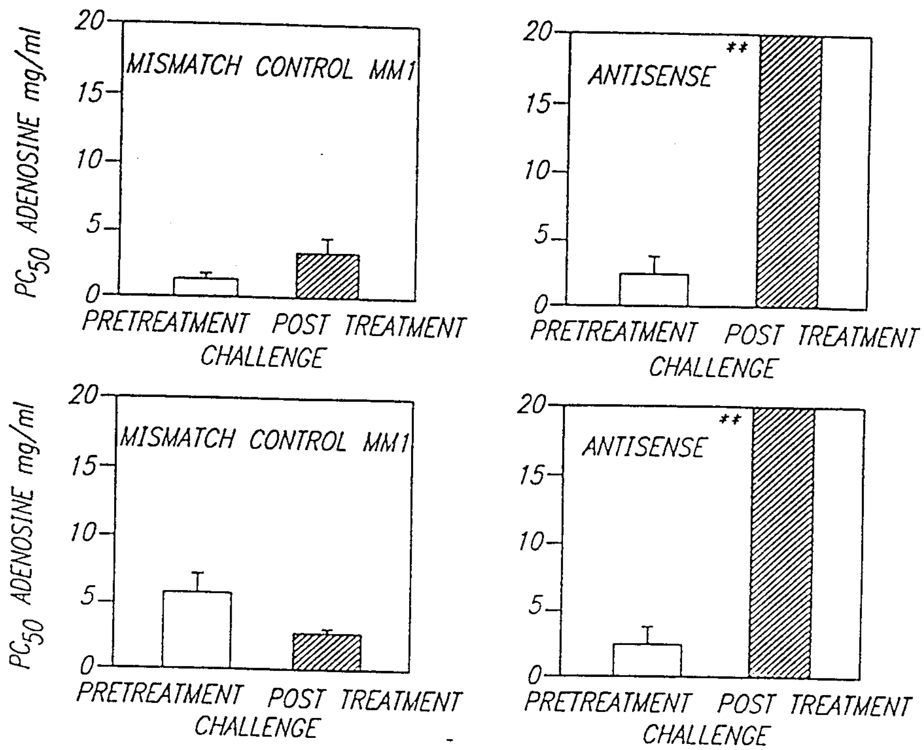


Figure 3

A_1MM CONTROL		A_1MM 2 CONTROL		A_1AS	
PRE ODN	POST ODN	PRE ODN	POST ODN	PRE ODN	POST ODN
3.56± 1.02	3.25±0.34	2.46±0.50	2.81±0.70	2.36±0.68	>19.5±0.34**

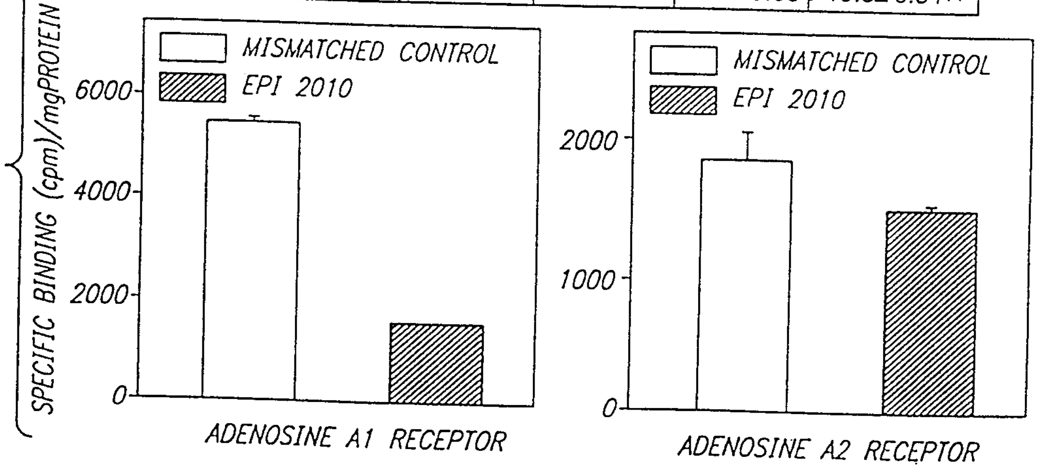


Figure 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/05643

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 31/70, 48/00, 49/00; C07H 21/00; C12N 15/63
US CL :514/44; 424/9.2; 536/22.1; 435/320.1, 375
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 424/9.2; 536/22.1; 435/320.1, 375

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NYCE, J.W. Insight into adenosine receptor function using antisense and gene-knockout approaches. Tips. February 1999, Vol. 20, No. 2, pages 79-83, see entire document.	1-41
Y	WO 96/40162 A1 (EAST CAROLINA UNIVERSITY) 19 December 1996, see entire document.	1-41
Y,P	US 5,994,315 A (NYCE ET AL) 30 November 1999, see entire document.	1-41

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
15 MAY 2000	11 JUL 2000

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Cynthia Lawrence for PETER BRUNOVSKIS, PH.D.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT	International application No. PCT/US00/05643

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN, MEDLINE, CAPLUS, BIOSIS, EMBASE, BIOSIS, SCISEARCH, WEST, USPT
search terms: antisense(s)oligonucleotide, adenosine(3w)15% or 12% or low, low adenosine or few adenosine and oligonucleotide, nyce